Collected papers

submitted by Roger David Waigh

for the degree of Doctor of Science

of the University of Bath

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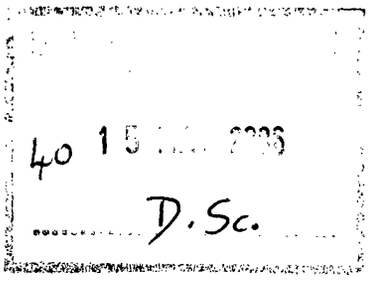
D. Sc.

Acknowledgements

Most of those I have to thank are co-authors of the papers which form this submission; I could not have done it without them and they know that. In addition there has been input from support staff in the Universities of Strathclyde and Manchester. You have to read the small print in the papers to find their names; thank you, one and all.

Support of various kinds was provided by my long-suffering wife, Sally, even to the point where she worked unpaid in the lab on various projects demanding different kinds of expertise. Most importantly, she has put up with my long hours, absences from home and sometimes plain exhaustion, with unfailing consideration.

To my children, Cathie and Tom; I'm sorry if I didn't spend as much time with you as I might have done when you were growing up, but you have both turned out pretty well!

Declaration

(*i*) I am the author, or one of the authors, of all the publications submitted.

(*ii*) The work described in papers 1,2,12,13 and 91 was carried out by me personally.

(*iii*) The work described in papers 5,9,10,11,14,15,16,17,18,19,21,22,23, 24,25,26,27,28, 29,33,50,55,68,69,70,71,72,73,97,99 and 100 was carried out largely by candidates for higher degrees of the Universities of Manchester and Strathclyde, under my sole supervision. In papers 37,38,39,40,41,42,45,47,48, 49,53, 54,62,63,64,65,67,75,77,78,79,80,81,82,83,84,85,86,89,90,93,94,95,96 and 98, I was joint supervisor of work carried out by candidates for such higher degrees. In papers 3,6,7,8 and 44 I was co-author of work submitted for a higher degree of the University of Bath; papers 31,32 at the University of Angers, France; similarly for paper 56 at the University of Sunderland. Paper 30 arose from work carried out under my sole supervision and at my instigation. Papers 35,36,58,59,60 and 61 arose from work for which I was a joint supervisor and instigator but no part of the work was included in any submission for any other award.

(*iv*) I used all or some of the work described in papers 1,2 and 4 as the basis for my successful submission for the degree of PhD of the University of Bath in 1969. The other submissions, by graduate students working under my sole or joint direction, or in which I was a co-author, are as follows:

Paper No.	Candidate	University	Degree awarded
3	N.Taylor	Bath	PhD
6	N.Taylor	Bath	PhD
7	N.Taylor	Bath	PhD
8	N.Taylor	Bath	PhD
9	D.Beaumont	Strathclyde	PhD
10	D.Beaumont	Strathclyde	PhD
11	D.Beaumont	Strathclyde	PhD
14	M.R.Euerby	Manchester	PhD
15	M.R.Euerby	Manchester	PhD
16	C.Mackay	Manchester	PhD
17	M.R.Euerby	Manchester	PhD
18	M.R.Euerby	Manchester	PhD
19	C.Mackay	Manchester	PhD
20	M.R.Euerby	Manchester	PhD
21	M.R.Euerby	Manchester	PhD
22	M.R.Euerby	Manchester	PhD
23	M.R.Euerby	Manchester	PhD
24	M.R.Euerby	Manchester	PhD
25	M.R.Euerby	Manchester	PhD
26	B.Cox	Manchester	PhD
27	J.P.Gavin	Manchester	PhD
28	T.A.Olugbade, S.P.Mackay	Manchester	PhD
29	M.R.Euerby, J.P.Gavin, T.A.Olugbade	Manchester	PhD
31	P.Pochet	Angers	PhD
32	C.Dartiguelongue	Angers	PhD
33	S.R.Bennett	Strathclyde	PhD
37	G.H.Dewar	Strathclyde	PhD
38	G.H.Dewar	Strathclyde	PhD
39	G.H.Dewar	Strathclyde	PhD

40	G.H.Dewar	Strathclyde	PhD
41	G.H.Dewar	Strathclyde	PhD
42	G.H.Dewar	Strathclyde	PhD
44	S.S.Patel	Bath	PhD
45	A.S.Hersom	Strathclyde	MSc
46	D.Beaumont	Strathclyde	PhD
47	T.Deeks	Manchester	PhD
48	T.Deeks	Manchester	PhD
49	T.Deeks	Manchester	PhD
51	T.A.Olugbade	Manchester	PhD
52	N.Silikas	Manchester	PhD
53	I.I.Hamdan	Strathclyde	PhD
54	I.I.Hamdan	Strathclyde	PhD
55	S.P.Mackay	Manchester	PhD
56	M.A.Kerry	Sunderland	PhD
62	A.I.Gray	Strathclyde	PhD
63	A.I.Gray	Strathclyde	PhD
64	A.I.Gray	Strathclyde	PhD
65	K.Panichpol	Strathclyde	MSc
66	A.I.Gray	Strathclyde	PhD
67	A.I.Gray	Strathclyde	PhD
68	A.O.Adeoye	Manchester	PhD
69	A.O.Adeoye	Manchester	PhD
70	G.A.Karikas	Manchester	PhD
71	G.A.Karikas	Manchester	PhD
72	B.Zerihun	Manchester	PhD
73	B.Zerihun	Manchester	PhD
75	A.A.Auzi	Strathclyde	PhD
77	D.M.N.Gakunju	Strathclyde	PhD
78	R.R.T.Majinda	Strathclyde	PhD
79	A.A.Auzi	Strathclyde	PhD
80	A.A.Auzi	Strathclyde	PhD
81	T.C.Fleischer	Strathclyde	PhD
82	R.R.T.Majinda	Strathclyde	PhD
83	A.A.Auzi	Strathclyde	PhD
84	T.C.Fleischer	Strathclyde	PhD
85	Z.Latif	Strathclyde	PhD
86	Z.Latif	Strathclyde	PhD
89	R.E.Lythgoe	Manchester	MSc
90	J.A.Shepherd	Manchester	PhD
93	P.J.Collier	Manchester	PhD
94	A.J.Ferdous	Manchester	PhD
95	S.J.Anie	Manchester	PhD
96	S.J.Anie	Manchester	PhD
97	A.J.Ferdous	Manchester	PhD
98	R.Blundell, D.Bloor	Manchester	PhD
99	T.Plumridge	Strathclyde	PhD
100	T.Plumridge	Strathclyde	PhD

19/12/02

Summary

The work which is described in these papers is presented in chronological order, in each of five connected areas. The overall theme is the discovery of new drug molecules and an understanding of how they work.

In section (1), the primary focus is on the development of new methods for the synthesis of isoquinolines, developing towards the synthesis of natural product analogues which have anticancer activity. This led directly to much of the work in section (3).

In section (2), the chemical synthesis of isoquinolines is developed in the specialist area of neuromuscular blocking agents. These culminate in the synthesis of atracurium, which is widely used as a surgical muscle relaxant.

In section (3) a variety of ideas are explored in the general context of drug discovery, many associated with isoquinolines. This section includes antimalarials and antileukaemics (for example benzo[c]phenanthridines) with biological activity mediated via DNA, as well as the development of new assay methods for DNA binding. The most recent work is focused on the discovery of new DNA-binding compounds, analogues of natural products, with potential anticancer and antiparasitic activity

Section (4) is concerned with the isolation and structure elucidation of a variety of natural products. This section cross-relates to sections (1), (2) and (3) in the isolation and identification of the benzo[c]phenanthridine alkaloid nitidine, which is a potent antimalarial.

The final section is concerned with the mechanisms of action and solution behaviour of drug molecules. A major theme in this group is the use of NMR, which reflects the methodology used in most of the other work.

Contents

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1. D.N. Harcourt and R.D. Waigh. A new synthesis of 4-oxo-1,2,3,4-tetrahydroisoquinolines. *Chem. Comm.*, 692 (1968) 1
2. D.N. Harcourt and R.D. Waigh. Synthesis of isoquinolines from benzyl aminonitriles. Part 1. *J. Chem. Soc. (C)*, 967-70 (1971) 2
3. D.N. Harcourt, N. Taylor and R.D. Waigh. Rearrangement in the synthesis of isoquinolines from benzylaminoacetonitriles. *J.C.S. Chem. Comm.*, 643-5 (1972) 6
4. J.R. Brooks, D.N. Harcourt and R.D. Waigh. Cyclisation of N-prop-2-ynyl benzylamines. Part 2. *J.C.S. Perkin 1*, 2588-91 (1973) 9
5. D.A. Livingstone and R.D. Waigh. Synthesis of 3-arylisoquinolines by thermolysis of 3-aryl-1,2-dihydroisoquinolin-4(3)-one salts. *J.C.S. Chem. Comm.*, 1026-7 (1978) 13
6. D.N. Harcourt, N. Taylor and R.D. Waigh. Cyclisation of benzylaminonitriles. Part 2. Evidence for two mechanisms of cyclisation. *J.C.S. Perkin 1*, 722-5 (1978) 15
7. D.N. Harcourt, N. Taylor and R.D. Waigh. Cyclisation of benzylaminonitriles. Part 3. Synthesis of 3-imidazolines. *J. Chem. Research* (S) 154, (M) 1954-74 (1978) 19
8. D.N. Harcourt, N. Taylor and R.D. Waigh. Cyclisation of benzylaminonitriles. Part 4. Rearrangement with cyclisation to a benzyl or phenethyl substituent. *J.C.S. Perkin 1*, 1330-33 (1978) 20
9. D. Beaumont and R.D. Waigh. Oxidative C-debenzylation of N-benzylpapaverinium bromide under mild alkaline conditions. *Chem. and Ind.*, 808 (1978) 24
10. D. Beaumont and R.D. Waigh. Bischler-Napieralski cyclisation of N-[2-(2-naphthyl)-ethyl]amides. *J. Chem. Research*, (S) 332, (M) 3864-77 (1979) 25
11. D. Beaumont and R.D. Waigh. Amine substitution in quaternary isoquinolinium salts. *Chem. and Ind.*, 291-2 (1980) 26
12. R.D. Waigh. Rearrangement of benzylaminonitriles to give cyclohepta[c]-pyrrol-6-ones. *J.C.S. Chem Comm.*, 1164-5 (1980) 28
13. R.D. Waigh. Differentiation of isomeric 1,2-dihydroisoquinolin-4(3H)-ones by proton magnetic resonance of their 4-benzyl derivatives. *Org. Magn. Reson.*, **13**, 310-11 (1980) 30
14. M.R. Euerby and R.D. Waigh. A convenient synthesis of 3-methylthiobenzaldehyde. *Synth. Comm.*, **11**, 849-51 (1981) 32
15. M.R. Euerby and R.D. Waigh. Cyclisation of benzylaminonitriles. Part 5. Alkylthio and arylthio activating groups. *J. Chem. Research*, (S) 240-1, (M) 2417-2460 (1982) 34
16. C. Mackay and R.D. Waigh. Rearrangement and cyclisation of N-(2-hydroxy-phenethyl)-2-aminoethylthiophens. *J.C.S. Chem. Comm.*, 793-4 (1982) 36
17. M.R. Euerby and R.D. Waigh. Rearrangement of N-(4-methoxybenzyl)-1-phenyl-2-aminoethanol to give 4-aryltetrahydroisoquinolines and a diarylethylamine. *Chem. and Ind.*, 287-8 (1983) 38
18. M.R. Euerby and R.D. Waigh. Methylthio activating groups in the synthesis of isoquinolines. *J.C.S. Chem. Comm.*, 127-8 (1984) 40
19. C. Mackay and R.D. Waigh. A new synthesis of 1,2-dihydro-□-carbolin-4(3H)-ones. *Heterocycles*, **22**, 687-9 (1984) 42
20. M.R. Euerby, R.D. Waigh, I.H. Hillier and J.P. Huke. A theoretical study of the site selectivity of methylthio and methoxy activating groups in the synthesis of isoquinolines. *J.C.S. Perkin II*, 1151-53 (1985) 45
21. M.R. Euerby, A.C. Oldroyd and R.D. Waigh. Synthesis of 4,5-dihydro-1H-imidazoles from 1,5-dihydro-2H-imidazoles using nickel boride. *Heterocycles*, **24**, 2871-77 (1986) 48
22. M.R. Euerby and R.D. Waigh. A convenient procedure for the reductive desulphurisation of thioethers with nickel boride. *Synth. Comm.*, **16**, 779-84 (1986) 55
23. M.R. Euerby and R.D. Waigh. Regioselectivity in the synthesis of isoquinolines with methoxy activating groups. *J. Chem. Research*, (S) 36-37, (M) 501-526 (1987) 58
24. M.R. Euerby and R.D. Waigh. The synthesis of isoquinolines using methylthio activating groups. *J. Chem. Research*, (S) 38-9 (M) 527-553 (1987) 60
25. M.R. Euerby and R.D. Waigh. Methylthio activating groups in the synthesis of tetrahydroisoquinolines and tetrahydro-2-benzazepines from N-allyl- and N-cinnamyl-benzylamines. *J. Chem. Research*, (S) 40-41 (M) 554-575 (1987) 62
26. B. Cox and R.D. Waigh. A modified synthesis of 4-chromanones. *Synthesis*, 709-10 (1989) 64
27. J.P. Gavin and R.D. Waigh. Cyclisation of benzylaminonitriles. Part 7. Regiospecific

formation of methoxy-substituted isoquinolin-4-ones using methylthio activating groups. *J.C.S. Perkin 1*, 503-8 (1990) 66
28. T.A. Olugbade, R.D. Waigh and S.P. Mackay. A very short route to fully aromatic 2,3,8,9- and 2,3,8,9,12- oxygenated benzo[c]phenanthridines. *J.C.S. Perkin 1*, 2657-2660 (1990) 72
29. M.R. Euerby, J.P. Gavin, T.A. Olugbade, S.P. Patel and R.D. Waigh. Cyclisation of benzylaminonitriles. Part 8. Anhydrous hydrogen fluoride as cyclising agent. *J. Chem. Research, (*S) 58-59, (M) 545-554 (1991) 76
30. O. Duval and R.D. Waigh. Improved regioselectivity of lithiation of methoxy benzaldehydes. *Synth. Comm.*, **21**, 43-48 (1991) 78
31. M.A. Lynch, O. Duval, P. Pochet, R.D. Waigh, High-yielding total synthesis of fagaronine chloride. *Bull. Soc. Chim. Fr.*, **131**, 718-722 (1994) 82
32. C. Dartiguelongue, S. Payan, O. Duval, L.M. Gomes, R.D. Waigh, Synthesis of □-amino esters using the Reformatsky reaction with □-amino nitriles. *Bull. Soc. Chim. Fr.* **134**, 769-772 (1997) 87
33. S.R. Bennett, A.R. Kennedy, A.I. Khalaf, R.D. Waigh, 2,8-Dimethyl-phenoxathiin 10-Oxide. *Acta Cryst.* **C54**, 1511-1513 (1998) 91

34. J.B. Stenlake, W.D. Williams, N.C. Dhar, R.D. Waigh and I.G. Marshall. Neuro-muscular blocking agents. The preparation and properties of *cis* and *trans* norcoralydine methiodides. *Eur. J. Med. Chem.*, **9**, 243-8 (1974) 94
35. J.B. Stenlake, J. Urwin, R.D. Waigh and R. Hughes. Biodegradable neuromuscular blocking agents I. Quaternary esters. *Eur. J. Med. Chem.*, **14**, 77-84 (1979) 100
36. J.B. Stenlake, J. Urwin, R.D. Waigh and R. Hughes. Biodegradable neuromuscular blocking agents II. Quaternary ketones. *Eur. J. Med. Chem.*, **14**, 85-88 (1979) 108
37. J.B. Stenlake, R.D. Waigh, J. Urwin, G.H. Dewar and R. Hughes. Biodegradable neuro-muscular blocking agents III. Bisquaternary esters. *Eur. J. Med. Chem.*, **16**, 508-14 (1981) 112
38. J.B. Stenlake, R.D. Waigh, J. Urwin, G.H. Dewar and R. Hughes. Bis-3,4-dihydroiso-quinolinium salts as potential short-acting neuromuscular blocking agents. *Eur. J. Med. Chem.*, **16**, 503-7 (1981) 119
39. J.B. Stenlake, R.D. Waigh, G.H. Dewar, R. Hughes, D. Chapple and G.G. Coker. Biodegradable neuromuscular blocking agents IV. Atracurium besylate and related polyalkylene di-esters. *Eur. J. Med. Chem.*, **16**, 513-24 (1981) 124
40. G.G. Coker, G.H. Dewar, R. Hughes, T.M. Hunt, J.P. Payne, J.B. Stenlake and R.D. Waigh. A preliminary assessment of Atracurium, a new competitive neuromuscular blocking agent. *Acta Anaesth. Scand.*, **25**, 67-9 (1981) 134
41. J.B. Stenlake, R.D. Waigh, G.H. Dewar, R. Hughes and D. Chapple. Biodegradable neuromuscular blocking agents. Part 5. □.□-bisquaternary polyalkylene phenolic esters. *Eur. J. Med. Chem.*, **18**, 273-6 (1983) 137
42. J.B. Stenlake, R.D. Waigh, G.H. Dewar, N.C. Dhar, R. Hughes, D.J. Chapple, J.C. Lindon, A.G. Ferrige and P.M. Cobb. Biodegradable neuromuscular blocking agents. Part 6. Stereochemical studies on atracurium and related polyalkylene diesters. *Eur. J. Med. Chem.*, **19**, 441-50 (1984) 141
43. R.D. Waigh. The chemistry behind atracurium. *Chem. Brit.*, 1209-12 (1988) 151
44. G.H. Dewar, I.G. Marshall, S.S. Patel and R.D. Waigh. Chemodegradable neuro-muscular blocking agents. Bis-(3-benzyl-3,4-dihydroisoquinolinium) salts. *Pharm. Sci. Comm.*, **4**, 3-14 (1993) 155
45. I.W. Rodger, A.S. Hersom and R.D. Waigh. Actions of two dopamine derivatives at adreno- and cholinoceptors. *J. Med. Chem.*, **22**, 117-19 (1979) 167
46. D. Beaumont, R.D. Waigh, M. Sunbhanich and M.W. Nott. Synthesis of 1-amino-methyl-1,2,3,4-tetrahydroisoquinolines and their actions at adrenoceptors both *in vivo* and *in vitro*. *J. Med. Chem.*, **26**, 507-15 (1983) 170
47. T. Deeks, P.A. Crooks and R.D. Waigh. Synthesis and analgesic properties of two leucine enkephalin analogues containing a conformationally restrained N-terminal tyrosine residue. *J. Med. Chem.*, **26**, 762-5 (1983) 179
48. W.J. Layton, S.L. Smith, P.A. Crooks, T. Deeks and R.D. Waigh. Synthesis and stereochemical analysis of 2-amino-1,2,3,4-tetrahydro-1,4-methano-naphthalene-2-carboxylic acid, a conformationally rigid phenylalanine derivative. *J.C.S. Perkin 1*, 1283-7 (1984) 183

49. T. Deeks, P.A. Crooks and R.D. Waigh. Leucine enkephaline analogues containing a conformationally restrained N-terminal amino acid residue. *J. Pharm. Sci.*, **73**, 457-60 (1984) 188
50. D.L.C. McCall, J. Alexander, J.Barber, R.G. Jaouhari, A. Satoskar and R.D. Waigh. The first protoberberine alkaloid analogue with *in vivo* antimalarial activity. *Bioorg. Med. Chem. Lett.*, **4**, 1663-1666 (1994) 192
51. T.A. Olugbade and R.D. Waigh. Synthetic benzo[c]phenanthridines with antileukaemic activity in mice. *Pharm. Sci.*, **2**, 259-264 (1996). 196
52. N. Silikas, D.L.C. McCall, D. Sharples, W.M. Watkins, R.D. Waigh and J. Barber. The antimalarial activity of berberine and some synthetic analogues. *Pharm. Sci.*, **2**, 55-58, (1996). 202
53. I.I. Hamdan, G.G. Skellern and R.D. Waigh, Separation of $pd(GC)_{12}$ from $pd(AT)_{12}$ by free solution capillary electrophoresis. *J. Cromatogr. A.*, **806**, 165-168 (1998) 206
54. I.I. Hamdan, G.G. Skellern and R.D. Waigh. Use of capillary electrophoresis in the study of ligand-DNA interactions. *Nucleic Acids Res.*, **26**, 3053-3058 (1998) 209
55. S.P. Mackay, L. Comoe, B. Desoize, O. Duval, J-C. Jardillier and R.D. Waigh. The effect of 12-alkoxy modification on the in vitro antileukaemic activity of *N*-methyl-2,3,8,9-tetramethoxybenzo[c]phenanthridinium salts. *Anti-Cancer Drug Design*, **13**, 797-813 (1998) 215
56. M.A. Kerry, O. Duval, R.D. Waigh and S.P. Mackay. The role of the iminium bond in the inhibition of reverse transcriptase by quaternary benzophenanthridines. *J. Pharm. Pharmacol.*, **50**, 1307-1315 (1998) 232
57. A. Ianoul, F. Fleury, O. Duval, R.D. Waigh, J.C. Jardillier, A.J.P. Alix, I. Nabiev. DNA binding by fagaronine and ethoxidine, inhibitors of human DNA topoisomerases I and II, probed by SERS and flow linear dichroism spectroscopy, *J. Phys. Chem.* (B), **103**, 2008- 2013, 1999 241
58. R.V. Fishleigh, K.R. Fox, A.I. Khalaf, A.R. Pitt, M. Scobie, C.J. Suckling, J. Urwin, R.D. Waigh and S.C. Young. DNA binding, solubility and partitioning characteristics of extended lexitropsins, *J. Med. Chem.*, **43**, 3257-3266 (2000) 247
59. A.I. Khalaf, A.R. Pitt, M. Scobie, C.J. Suckling, J. Urwin, R.D. Waigh, R.V. Fishleigh, S.C. Young and W.A. Wylie, The synthesis of some head to head linked DNA minor groove binders, *Tetrahedron*, 46, 5225-5239 (2000) 257
60. A.I. Khalaf, A.R. Pitt, M. Scobie, C.J. Suckling, J. Urwin, R.D. Waigh, R.V. Fishleigh and S.C. Young. Synthesis of novel DNA binding agents: indole-containing analogues of bis-netropsin, *J. Chem. Research* (S) 264-265 (M) 751-770 (2000) 272
61. A.I. Khalaf, R.G. Alvarez, C.J. Suckling and R.D. Waigh. Unexpected dealkylation during nucleophilic substitution: synthesis of 2-N,N-dialkylamino benzoxazoles and benzothiazoles. *Tetrahedron* **56**, 8567-8571 (2000) 274

62. A.I. Gray, R.D. Waigh and P.G. Waterman. A method for the determination of substitution pattern in coumarins using a lanthanide shift reagent. *J.C.S. Chem. Comm.*, 632-3 (1974) 279
63. A.I. Gray, R.D. Waigh and P.G. Waterman. Avicennol, a new pyranocoumarin from the rootbark of *Zanthoxylum avicennae*, and its conversion into avicennin. *J.C.S. Perkin 1*, 487-491 (1975) 281
64. F. Fish, A.I. Gray, R.D. Waigh and P.G. Waterman. Dipetalolactone: a novel pyrano-coumarin from the rootbark of *Zanthoxylum dipetalum*. *Phytochemistry*, **15**, 313-6 (1976) 285
65. K. Panichpol, R.D. Waigh and P.G. Waterman. Chondrofoline from *Uvaria ovata*. *Phytochemistry*, **16**, 621-2 (1977) 289
66. A.I. Gray, R.D. Waigh and P.G. Waterman. Cis-avicennol, a new pyranocoumarin from the rootbark of *Zanthoxylum elephantiasis*. *Phytochemistry*, **16**, 1017-8 (1977) 291
67. A.I. Gray, R.D. Waigh and P.G. Waterman. Interactions of coumarins with a lanthanide shift reagent. *J.C.S. Perkin II*, 391-5 (1978) 293
68. A.O. Adeoye and R.D. Waigh. Secoiridoid and triterpenic acids from the stems of *Nauclea diderrichii*. *Phytochemistry*, **22**, 975-8 (1983) 298
69. A.O. Adeoye and R.D. Waigh. Desoxycordifolinic acid from *Nauclea diderrichii*. *Phytochemistry*, **22**, 2097-8 (1983) 302
70. G.A. Karikas, M.R. Euerby and R.D. Waigh. Constituents of the stems of *Arbutus unedo*. *Planta Med.*, 223-4 (1987) 304
71. G.A. Karikas, M.R. Euerby and R.D. Waigh. Isolation of piceoside from *Arctostaphylos uva-ursi*. *Planta. Med.*, 307-8 (1987) 306
72. R.D. Waigh, B. Zerihun and M.R. Euerby. Three diterpenes with a secolabdane

skeleton from *Clutia abyssinica. Phytochemistry*, **29**, 2935-2938 (1990) 308
73. R.D. Waigh, B. Zerihun and D.J. Maitland. Ten 5-methyl coumarins from *Clutia abyssinica. Phytochemistry*, **30**, 333-335 (1991) 312
74. R.J. Nash, M. Rothschild, E.A. Porter, A.A. Watson, R.D. Waigh and P.G. Waterman. Calystegines in *Solanum* and *Datura* species and the death's head hawkmoth (*Acherontia atropus*). *Phytochemistry*, **34**, 1281-1283 (1993) 315
75. A.A. Auzi, A.I. Gray, T.G. Hartley, R.D. Waigh and P.G. Waterman. A xanthine alkaloid from the leaves of *Bosistoa floydii. Phytochemistry*, **36**, 535-536 (1994) 318
76. R.J. Nash, P.I. Thomas, R.D. Waigh, G.W.J. Fleet, M.R. Wormald, P.M. de Q. Lilley and D.J. Watkin, Casaurine : A very highly oxygenated pyrrolizidine alkaloid. *Tetrahedron Lett.*, **35**(42), 7849-7852 (1994) 320
77. D.M.N. Gakunju, E.K. Mberu, S.F. Dossaji, A.I. Gray, R.D. Waigh, P.G. Waterman and W.M. Watkins, Potent antimalarial activity of the alkaloid nitidine, isolated from a Kenyan herbal remedy. *Antimicrob. Agents Chemother.*, **39**, 2606-9 (1995) 324
78. R.R.T. Majinda, A.I. Gray, R.D. Waigh and P.G. Waterman. A *seco*-olean-18-ene triterpene acid from *Vahlia capensis. Phytochemistry*, **38**, 461-463 (1995) 328
79. A.A. Auzi, T.G. Hartley, R.D. Waigh and P.G. Waterman. Acridone alkaloids from *Bosistoa Transversa. Phytochemistry*, **42**, 235-238 (1996) 331
80. A.A. Auzi, P.I. Forster, T.G. Hartley, R.D. Waigh and P.G. Waterman. Phloroglucinol derivatives from leaves of *Bosistoa pentacocca. Phytochemistry*, **45**(8), 1673-1678 (1997). 335
81. T.C. Fleischer, R.D. Waigh and P.G. Waterman. Bisabolene Sesquiterpenes and Flavanoids from *Friesodielsia Enghiana. Phytochemistry*, **44**, 315-318 (1997). 341
82. R.R.T. Majinda, R.D. Waigh and P.G. Waterman. Bufadienolides and Other Constituents of *Urginea sanguinea. Planta Med.*, **63**, 188-190 (1997) 345
83. A.A. Auzi, T.G. Hartley, R.D. Waigh and P.G. Waterman. Novel prenylated acetophenones from *Bosistoa Selwynii. Nat. Prod. Lett.*, **11**, 137-144 (1998) 348
84. T.C. Fleischer, R.D. Waigh and P.G. Waterman. A novel retro-dihydrochalcone from the stem bark of *Uvaria mocoli. Phytochemistry*, **47**, 1387-1391 (1998) 356
85. Z. Latif, T.G. Hartley, M.J. Roce, R.D. Waigh and P.G. Waterman. Isobutylamides and Coumarins fom *Melicope melanophloia. Biochem. Syst. Ecol.*, **26**, 467-468 (1998) 361
86. Z. Latif, T.G. Hartley, M.J. Roce, R.D. Waigh and P.G. Waterman. Novel and Insecticidal isobutylamides from *Dinosperma erythrococcoa. J. Nat. Prod.*, **61**(5), 614-619 (1998) 363

87. D. Attwood, S.P. Agarwal and R.D. Waigh. Effect of the nature of the hydrophobic group on the mode of association of amphiphilic molecules in aqueous solution. *J.C.S. Faraday 1*, **76**, 2187-93 (1980) 369
88. L. Aarons, D. Bell, R.D. Waigh and Q. Yee. Parabolic structure-activity relationships: a simple pharmacokinetic model. *J. Pharm. Pharmacol.*, **34**, 746-49 (1982) 376
89. N.A. Dickinson, R.E. Lythgoe and R.D. Waigh. Some observations on the WATR method for water suppression at 80MHz. *Magn. Reson. Chem.*, **25**, 996-7 (1987) 380
90. J. A. Shepherd, R.D. Waigh and P. Gilbert. Antibacterial action of 2-bromo-2-nitropropan-1,3-diol (bronopol). *Antimicrob. Agents Chemother.*, **32**, 1693-98 (1988) 382
91. R.D. Waigh. Peak selection through orthogonal focusing. *J. Magn. Reson.*, **80**, 306-8 (1988) 388
92. R.D. Waigh and P. Gilbert. Mechanisms of chemical reactions with biomolecules. In "Mechanisms of antibacterial action: their study and exploitation.", eds S.P. Denyer and W.B. Hugo, SAB Technical Series No 26, 251-262 (1990) 391
93. P.J. Collier, A. Ramsey, R.D. Waigh, K.T. Douglas, P. Austin and P. Gilbert. Chemical reactivity of some isothiazolone biocides. *J. Appl. Bact.*, **69**, 578-584 (1990) 403
94. A.J. Ferdous, N.A. Dickinson and R.D. Waigh. ^{1}H NMR as an analytical tool for the investigation of hydrolysis rates: a method for the rapid evaluation of the shelf-life of aqueous solutions of drugs with ester groups. *J. Pharm. Pharmacol.*, **43**, 860-862 (1991) 410
95. S.J. Anie, J.T. Fell and R.D. Waigh. Visualisation of a fluorinated pharmaceutical formulation in the anaesthetised rat using ^{19}F and ^{1}H-NMR imaging. *Int. J. Pharmaceutics*, **69**, 181-186 (1991) 413
96. S.J. Anie, J.T. Fell, R.D. Waigh and B. Wood. Gastrointestinal transit studies in the rat using ^{19}F NMR. *Int. J. Pharmaceutics*, **76**,183-185 (1991) 419
97. A.J. Ferdous and R.D. Waigh. Application of the WATR technique for water suppression in 1H NMR spectroscopy in determination of the kinetics of hydrolysis of

neostigmine bromide in aqueous solution. *J. Pharm. Pharmacol.*, **45**, 559-562 (1993) 422
98. D. Attwood, R.D. Waigh, R. Blundell, D. Bloor, A. Thevand, E. Boitard, J.-P. Dubes and H. Tachoire. ^{1}H and ^{13}C NMR studies of the self-association of chlorpromazine hydrochloride in aqueous solution. *Magn. Reson. Chem.*, **32**, 468-472 (1994) 426
99. T.H. Plumridge, G. Steele and R.D. Waigh. Geometry-based simulation of the hydration of small molecules. *PhysChemComm*, 8, 2000 431
100. T.H.Plumridge and R.D.Waigh. Water structure theory and some implications for drug design. J. Pharm. Pharmacol., 54, 1155-1179, 2002 437

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A New Synthesis of 4-Oxo-1,2,3,4-tetrahydroisoquinolines

By D. N. HARCOURT* and R. D. WAIGH

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DURING some synthetic work on 3-arylisoquinolines, we prepared certain 4-oxo-1,2,3,4-tetrahydroisoquinolines. None of the reported routes[1,2] possessed the flexibility that we sought, with the exception of the unsuccessful attempt[3] to cyclise α-amino-nitriles of the type (I), which are formed in good yield by a modified Strecker synthesis,[4] and potentially afford ready variation in substitution at the C-3 position of the isoquinoline. In view of the recorded[2] instability of simple 4-oxo-isoquinolines, we considered this route warranted investigation, and found the mild conditions for the cyclisation of a number of nitriles (I; R^1 = OMe). The nitrile (2 g.) was dissolved in concentrated sulphuric acid (10 ml.) and heated at 50° for 4 hr., or left overnight at room temperature. Dilution followed by basification with 5N sodium hydroxide gave the product (II) in good yield and a high state of purity.

4-Oxo-1,2,3,4-tetrahydroisoquinolines (II; R^1 = OMe)

R^2	R^3	% yield	m.p.† (°C)
Ph	H	53	138
Ph	Me	83	150
Me	Me	60	135
$CH_2{\cdot}[CH_2]_3{\cdot}CH_2$		80	147

† Determined for analytical sample on a Kofler hot-stage.

Structural assignment of the isoquinolines is based upon elemental analysis and diagnostic i.r. and n.m.r. spectra.

Some difficulty was encountered in the isolation of the free base (II; R^1 = OMe, R^2 = Ph, R^3 = H) and best yields were obtained by isolation as the hydrochloride. We are further preparing the 3-mono- and 3-un-substituted 4-oxo-1,2,3,4-tetrahydroisoquinolines in view of the low yields recently reported[5] for the cyclisation of *N*-benzylglycine esters in the 6,7-dimethoxy-series.

(I) (II)

Attempted cyclisation of amino-nitriles (I; R^1 = H) has so far resulted in failure; with the conditions described here, conversion to the corresponding amide occurs.

We thank the Pharmaceutical Society of Great Britain for a research scholarship (to R.D.W.).

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[1] Neth. Pat. 6,504,208/1965; T. Kametani and K. Fukumoto, *J. Chem. Soc.*, 1963, 4289; N. Itoh and S. Sugasawa, *Tetrahedron*, 1959, **6**, 16.
[2] I. G. Hinton and F. G. Mann, *J. Chem. Soc.*, 1959, 599.
[3] B. B. Dey and T. R. Govindachari, *Arch. Pharm.*, 1937, **275**, 383.
[4] R. B. Wagner and H. D. Zook, "Synthetic Organic Chemistry," Wiley, New York, 1953, p. 605 and references cited.
[5] G. Grethe, H. L. Lee, M. Uskokovic, and A. Brossi, *J. Org. Chem.*, 1968, **33**, 491.

Synthesis of Isoquinolines from Benzylaminoacetonitriles. Part I. Compounds prepared from Veratrylamine [1]

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3,4-Dimethoxybenzylaminoacetonitriles cyclise in concentrated sulphuric acid to give, after hydrolysis, 1,2-dihydroisoquinolin-4(3*H*)-ones, in excellent yield. 3,3-Disubstituted 1,2-dihydroisoquinolin-4(3*H*)-ones are stable as the free base, in contrast to the 3-unsubstituted and 3-monosubstituted analogues. 1,2-Dihydro-6,7-dimethoxy-3-phenylisoquinolin-4(3*H*)-one as the free base underwent aerial oxidation to give 6,7-dimethoxy-3-phenylisoquinolin-4-ol, and could also be converted by a series of reactions into 6,7-dimethoxy-3-phenylisoquinoline in good yield.

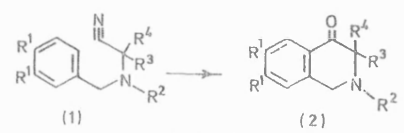
A MUCH-QUOTED review of twenty years ago [2] placed emphasis on the difficulty of synthesis of isoquinolines by routes analogous to the Pomeranz–Fritsch reaction. Many failures were reported,[3] and an attempt was made to rationalise the situation in general terms.[4]

Since then, the work of several groups [5–8] has altered the picture fundamentally. It is now apparent that the benzylamine route has no inherent theoretical barriers, but that the primary product is often difficult to isolate. The 1,2-dihydro-, 4-hydroxytetrahydro-, and 4-oxotetrahydro-isoquinolines, usual products of this kind of reaction, are all now known [7,9,10] to be relatively unstable. Thus the reduction of a reactive isoquinoline intermediate, without isolation, dramatically increased the yield of the corresponding tetrahydroisoquinoline.[6]

In view of the natural advantages of this approach to isoquinolines, particularly regarding the availability of starting materials, we were attracted to a reappraisal of one of the past unsuccessful routes. It was reported [3] that internal Hoesch reaction of the benzylaminonitrile (1a) with zinc chloride and dry hydrogen chloride in dry ether failed to give the required isoquinolinone (2a); other failures with similar systems under similar conditions were reported by other workers.[11] However, since α-aminonitriles are readily available,[12] this route potentially possesses great flexibility with respect to the 3-position of the isoquinoline (Scheme 1).

With a view to later extension to alkaloid syntheses, and in connection with other synthetic work,[13] we first studied the aminonitrile (1b), which would give a 3-phenylisoquinoline. Unfortunately, this compound and the corresponding tertiary base (1c) gave only the amide, formed by normal hydrolysis.

With the 'activated' analogue (1d) we were at first unsuccessful with a variety of acid treatments. We eventually succeeded in effecting cyclisation to compound (2d) by use of concentrated sulphuric acid, at first overnight at room temperature, and later, more conveniently,



(1) (2)

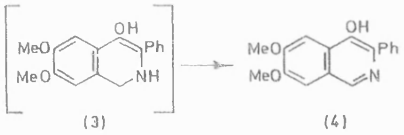
SCHEME 1

	R^1	R^2	R^3	R^4
a;	OMe	H	H	H
b;	H	H	Ph	H
c;	H	Me	Ph	H
d;	OMe	H	Ph	H
e;	OMe	H	Me	Me
f;	OMe	H	$[CH_2]_5$	
g;	OMe	H	Ph	Me
h;	OMe	H	DMP	H

DMP = 3,4-$(MeO)_2C_6H_3$

at 50 °C for 3·5—4 h. I.r., n.m.r., and analytical data for the product were unambiguous.

While the salts of the isoquinolinone produced were apparently stable indefinitely, in agreement with a previous report,[10] the free base underwent aerial oxidation on exposure to the air for several days, giving the aromatic isoquinolin-4-ol (4), possibly *via* the enolic form (3) (Scheme 2).



(3) (4)

SCHEME 2

To test both the enolisation hypothesis and the flexibility of our route, we attempted cyclisation of the

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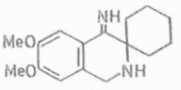
[1] Preliminary communication, D. N. Harcourt and R. D. Waigh, *Chem. Comm.*, 1968, 692.
[2] W. J. Gensler, *Org. Reactions*, 1951, **6**, 191.
[3] For example B. B. Dey and T. R. Govindachari, *Arch. Pharm.*, 1937, **275**, 383.
[4] J. Malan and R. Robinson, *J. Chem. Soc.*, 1927, 2653.
[5] R. Quelet and N. Vinot, *Compt. rend.*, 1957, **244**, 909; N. Vinot, *Ann. Chim.* (*France*), 1958, **3**, 461; N. Vinot and R. Quelet, *Bull. Soc. chim. France*, 1959, 1164.
[6] J. M. Bobbitt, K. L. Khanna, and J. M. Kiely, *Chem. and Ind.*, 1964, 1950; J. M. Bobbitt, J. M. Kiely, K. L. Khanna, and R. Ebermann, *J. Org. Chem.*, 1965, **30**, 2247.
[7] J. M. Bobbitt and J. C. Sih, *J. Org. Chem.*, 1968, **33**, 856.
[8] U.S.P. 357,318/1964; Dutch P. 6,504,208/1965; G. Grethe, H. L. Lee, M. Uskokovic, and A. Brossi, *J. Org. Chem.*, 1968, **33**, 491.
[9] H. Schmid and P. Karrer, *Helv. Chim. Acta*, 1949, **32**, 960.
[10] I. G. Hinton and F. G. Mann, *J. Chem. Soc.*, 1959, 599.
[11] S. H. Oakeshott and S. G. P. Plant, *J. Chem. Soc.*, 1927, 484; R. D. Haworth, W. H. Perkin, and J. Rankin, *ibid.*, 1925, 1444.
[12] For a review, see P. van Daele, *Mededel. vlaam. chem. Ver.*, 1961, **23**, 163.
[13] J. R. Brooks and D. N. Harcourt, *J. Chem. Soc.* (*C*), 1969, 625; D. N. Harcourt and R. D. Waigh, in preparation.

aminonitrile (1e). The expected product is incapable of enolisation, and was in fact found to be stable under all the conditions employed, being obtained crystalline from hot, strong sodium hydroxide solution.

In the case of the cyclohexanone derivative (1f) the initial product of the reaction, obtained by dilution of the concentrated sulphuric acid solution by direct addition to cold 5N-sodium hydroxide solution, was not the pure isoquinolinone (2f). The major component of the mixture gave an elemental analysis closer to that calculated for the imine (5); however it was not obtained



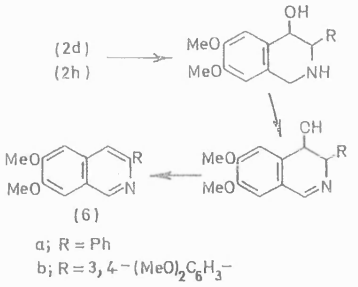
(5)

analytically pure. The i.r. and n.m.r. data of the mixture were ambiguous, but accurate mass measurement of the parent ion in the mass spectrum (*m/e* 274) indicated the molecular formula to be $C_{16}H_{22}N_2O_2$, consistent with the imine structure. Treatment of the imine with aqueous acid generated the expected ketone quickly and quantitatively. Similar experiences with imines from nitrile cyclisations have been recorded.[14] Another 3,3-disubstituted isoquinolinone (2g) was obtained in excellent yield, without difficulty.

With the 3-unsubstituted isoquinoline (2a), using direct basification of the sulphuric acid cyclisation medium without a preliminary dilution stage, we at first could obtain no product whatever. Presumably the intermediate imine is subject to attack by hydroxide, giving highly acid-, base-, and water-soluble product(s). However, when the concentrated acid was added carefully to ice–water, and set aside before basifying, the isoquinolinone could be isolated as the hydrochloride, after extraction with chloroform. The 30—50% yield represents a substantial improvement over that obtained by Grethe and his co-workers,[8] from cyclisation of the analogous glycine ester.

In order to investigate further the applicability of the route to alkaloid synthesis, we studied the cyclisation of the aminonitrile (1h). The expected product is potentially an intermediate for both benzo[*c*]phenanthridine and protoberberine types of ring system. Although the cyclisation succeeded, the low solubility of the product and by-product hydrochlorides in most solvents reduced the yield of pure material to 24%. No doubt this could be increased by improvements in the work-up technique.

The recent publication [15] of a simple synthesis of norcoralydine from 3-(3,4-dimethoxyphenyl)-6,7-dimethoxyisoquinoline has anticipated our own scheme. In order to realise the synthesis from our intermediate, it would be necesary to convert the dihydroisoquinolin-4-one (2h) into the corresponding aromatic isoquinoline (6b). As a model, we had chosen to convert the isoquinolinone (2d) (Scheme 3) into the aromatic isoquinoline (6a). Each of the stages is well established in principle,[16,17] and in fact the conversion was achieved in 44% overall yield.



(6)

a; R = Ph

b; R = 3,4-$(MeO)_2C_6H_3$-

SCHEME 3

We hope shortly to publish the results of cyclisations of other aminonitriles, including several with *N*-alkyl substituents, which have given different results.

EXPERIMENTAL

M.p.s of bases were taken with a Kofler hot-stage apparatus, and are corrected. M.p.s of salts (not corrected) were taken for samples in capillary tubes with a Gallenkamp apparatus; the tube was inserted 15—20° below the m.p. and heated at 5—7° per min. I.r. spectra were obtained with a Unicam SP 200 instrument for potassium bromide discs or liquid films. N.m.r. spectra were recorded with a Varian A60 instrument (tetramethylsilane as internal reference). Organic solutions were dried over anhydrous magnesium sulphate. Hydrochlorides were prepared by addition of hydrogen chloride in ether to an organic solution of the base.

Preparation of Aminonitriles.—General method. A solution of 3,4-dimethoxybenzylamine (0·1 mol) in water (200 ml) was made just acid with dilute hydrochloric acid. The aldehyde or ketone (0·1 mol), dissolved in ethanol (100 ml) if necessary, was added with stirring. Potassium cyanide (10 g) in water (50 ml) was added during 10 min with vigorous stirring; the solution was then stirred for 1 h and set aside overnight. The product was filtered off or extracted with ether or chloroform as appropriate, the solid or solution being washed thoroughly with water. Crystallisation, if necessary, or evaporation followed by crystallisation, gave the *aminonitrile* (see Tables 1 and 2). Where the m.p. and i.r. spectrum indicated a satisfactory degree of purity, the crude aminonitrile was used for cyclisation.

1,2-Dihydro-6,7-dimethoxyisoquinolin-4(3H)-ones.—General method. The aminonitrile (2 g) was dissolved with care in concentrated sulphuric acid (10 ml), so as to avoid excessive heating and consequent charring. The solution

[14] C. K. Bradsher and D. J. Beavers, *J. Org. Chem.*, 1956, **21**, 1067.

[15] N. L. Dutta, M. S. Wadia, and A. A. Bindra, *Indian J. Chem.*, 1969, **7**, 527.

[16] G. Grethe, H. L. Lee, M. Uskokovic, and A. Brossi, *J. Org. Chem.*, 1968, **33**, 494.

[17] W. G. D. Lugton, personal communication; M. Sainsbury D. W. Brown, S. F. Dyke, and G. Hardy, *Tetrahedron*, 1969, **25**, 1881.

was heated at 50° for 4 h; slight variations in time had little effect. No systematic effort was made to discover the best reaction conditions. Cyclisation overnight at room temperature gave slightly lower yields. The chilled solution was added cautiously to ice–water; more ice was added as necessary to maintain the temperature below 5°. The final

TABLE 1

Spectroscopic data for benzylaminoacetonitriles (1)

	$\nu_{max.}$/cm^{-1} (NH)	$\nu_{max.}$/cm^{-1} (C≡N)	^{1}H N.m.r. (δ values in p.p.m.; solvent $CDCl_3$)
a	3350	2230	
b	3350	2230	7 (10H, m), 4·3 (1H), 3·6 (2H), 1·8br (1H) *†
d	3340	2220	7·15 (5H, m), 6·7 (3H, m), 4·5 (1H), 3·7 (2H), 3·65 (6H), 1·9br (1H) †
e	3330	2230	6·9 (3H, m), 3·85 (8H, d), 1·5 (7H) †
f	3325	2225	6·9 (3H, m), 3·85 (8H, d), 1·65br (11H) †
g	3300	2230	7·5 (5H, m), 6·85 (3H, m), 3·75 (8H), 2·7br, (1H) † 1·75 (3H)
h	3350	2220	

* In CCl_4. † 1H exchangeable.

TABLE 2

Benzylaminoacetonitriles (1)

	Yield (%)	M.p. T/°C	Found (%) C	H	N	Required (%) C	H	N
a	66	181—185 *[a] (decomp.)	54·2	6·3	11·1	54·4	6·2	11·5
d	93	65 [b]	72·3	6·4	9·8	72·3	6·4	9·9
e	100	53 [c]	66·5	7·8	11·95	66·1	7·9	12·2
f	80	88 [c]	70·4	7·7	10·3	70·0	8·1	10·2
g	53	72—74 [c]	73·1	6·7	9·4	72·9	6·8	9·45
h	98	60—61 [c]	67·1	6·6	7·8	66·65	6·5	8·2

* Hydrochloride (lit.,[3] 188°). The free base had m.p. 58—62° (lit.,[8] 64°), after crystallisation from ether–petroleum (b.p. 40—60°) and m.p. 98—100° after crystallisation from benzene–petroleum (b.p. 60—80°).

[a] From ethanol. [b] From ether–petroleum (b.p. 80—100°). [c] From petroleum (b.p. 80—100°).

volume was about 200 ml. This solution was set aside for 30 min, and then basified carefully with 5N-sodium hydroxide or concentrated ammonium hydroxide, the addition of ice being continued as before. The *isoquinolinone* was

TABLE 3

Spectroscopic data for 1,2-dihydro-6,7-dimethoxyisoquinolin-4(3*H*)-ones (2)

	$\nu_{max.}$/cm^{-1} (NH)	$\nu_{max.}$/cm^{-1} (C=O)	^{1}H N.m.r. (δ values in p.p.m.; solvent $CDCl_3$)
a	3350	1660	7·5 (1H), 6·65 (1H), 4·05 (2H), 3·9 (6H,d), 3·5 (2H), 3·0br (1H) *
d	3350	1665	7·3 (1H), 7·1 (5H), 6·4 (1H), 4·4 (1H), 3·95 (2H), 3·8 (6H), 2·1br (1H) *
e	3340	1655	7·5 (1H), 6·6 (1H), 4·1 (2H), 3·9 (6H), 2·3 (6H), 2·3br (1H),* 1·3 (6H)
f	3300	1650	7·5 (1H), 6·65 (1H), 4·0 (2H), 3·9 (6H), 1·65 (10H, m) †
g	3300	1650	7·7 (1H), 7·4 (5H, m), 3·95 (5H), 3·85 (3H), 2·7br (1H),* 1·6 (3H)
h	2750 ‡	1680 ‡	

* 1H exchangeable. † Integral trace indicated 1H between 2·2 and 3·2 p.p.m. before shaking with deuterium oxide, but not afterwards. ‡ Hydrochloride.

filtered off or extracted with chloroform, and washed with water, before evaporating, if necessary, and drying. At this stage the isoquinolinones (2a) and 2(h) were converted

TABLE 4

1,2-Dihydro-6,7-dimethoxyisoquinolin-4(3*H*)-ones (2)

	Yield (%)	M.p. T/°C	Found (%) C	H	N	Required (%) C	H	N
a	30—50	235—236° *[a]						
d	60	138—139 [b]	71·8	6·2	4·8	72·0	6·05	4·9
e	53	135 [c]	66·4	7·3	6·1	66·4	7·3	5·95
f	80	147 [d]	70·0	7·8	5·3	69·8	7·7	5·1
g	83	150 [d]	72·8	6·3	4·7	72·8	6·45	4·7
h	24	255—256 †[e]	59·7	5·8	3·3	60·0	5·8	3·7

* Hydrochloride (lit.,[8] 236—237°). † Hydrochloride.

[a] From methanol. [b] From ether. [c] From benzene–petroleum (b.p. 80—100°). [d] From petroleum (b.p. 80—100°). [e] From water.

into the hydrochlorides, since the free bases are unstable. The need for such a procedure was indicated with the isoquinoline (2d), but it was more convenient to avoid basification of the diluted acid solution, since on prolonged refrigeration the solution deposited microcrystalline *1,2-dihydro-6,7-dimethoxy-3-phenylisoquinolin-4(3H)-one* (2d) *hydrogen sulphate*, which was stable and could be recrystallised from methanol–ether to give grey-green rods, m.p. 132—135° (decomp.), $\nu_{max.}$ 1680 cm^{-1} (Found: C, 54·0; H, 5·2; N, 3·55. $C_{17}H_{16}NO_7S$ requires C, 53·5; H, 5·0; N, 3·7%); yield 67%, after filtration from the mother liquor, washing with water, and drying.

1,2,3,4-Tetrahydro-4-imino-6,7-dimethoxyisoquinoline-3-spirocyclohexane (5).—The procedure adopted was as for the preparation of the isoquinolin-4-ones, except that the concentrated sulphuric acid solution after cyclisation was added, with caution, directly to ice–cold 10% sodium hydroxide. The white precipitate was filtered off, washed, and dried thoroughly (1·1 g; m.p. 123°). [This solid could be converted quantitatively into 1,2-dihydro-6,7-dimethoxyisoquinolin-4(3*H*)-one-3-spirocyclohexane, m.p. 148°, identical with that obtained previously, by dissolution in dilute hydrochloric acid and rebasification with sodium hydroxide.] After repeated crystallisation from petroleum (b.p. 80—100°) the solid had m.p. 125—126°, and probably contained about 20% of the isoquinolin-4-one (i.r. spectrum and elemental analysis). The major component was identified by accurate mass measurement of the parent ion in the mass spectrum (Found: M^+, 274·1682. $C_{16}H_{22}N_2O_2$ requires M, 274·1681).

6,7-Dimethoxy-3-phenylisoquinolin-4-ol (4).—1,2-Dihydro-6,7-dimethoxy-3-phenylisoquinolin-4(3*H*)-one (2d) (1·5 g) dissolved in 50% ether–chloroform (300 ml) was set aside for 3 days. Evaporation gave a pale brown gum, which on trituration with ether containing a little ethanol gave the *isoquinolin-4-ol* (0·55 g, 37%) as a pale yellow powder, m.p. 220°. A sample crystallised twice from aqueous ethanol as pale yellow plates had m.p. *ca.* 240°; final crystallisation from chloroform–petroleum (b.p. 40—60°) gave white microcrystals, m.p. 249°, $\nu_{max.}$ 3250 and 1625 cm^{-1}, δ ($CF_3{\cdot}CO_2H$) 8·7 (1H), 7·5 (7H), and 4·1 (6H, d) p.p.m. (Found: C, 72·6; H, 5·5; N, 5·1. $C_{17}H_{15}NO_3$ requires C, 72·6; H, 5·4; N, 5·0%).

6,7-Dimethoxy-3-phenylisoquinoline (6a).—1,2-Dihydro-6,7-dimethoxy-3-phenylisoquinolin-4(3*H*)-one (2d) hydrogen sulphate (30 g) dissolved in ethanol (300 ml) was basified carefully with sodium hydroxide, and sodium borohydride (10 g) was added. Next day the excess of borohydride was destroyed with dilute acetic acid, and the solution was rebasified with sodium hydroxide and extracted with chloroform. Drying and evaporation of the extract yielded a

brown glass, which on trituration with ethanol–ether gave a yellow solid (19 g), m.p. 134—144°, clearly a mixture of bases, presumably the diastereoisomers of the tetrahydroisoquinolin-4-ol and their dehydration product.

The mixed bases (16·5 g) were dissolved in chloroform (200 ml) and *N*-bromosuccinimide (10·7 g) was added with swirling. After 2 h the solution was diluted with ether (800 ml.), and the green amorphous precipitate (23 g.) was filtered off and washed with ether. The green solid was suspended in concentrated hydrochloric acid (100 ml) and heated on a boiling water bath for 30 min. The cooled solution was diluted with water (400 ml) and the solid was filtered off, suspended in water, and treated with sodium hydroxide and ethanol, to give *6,7-dimethoxy-3-phenylisoquinoline* (6a) as a brown crystalline solid, m.p. 127° (8 g, 44% based on isoquinolinone hydrogen sulphate), $\nu_{max.}$ 1620 cm^{-1}, δ ($CDCl_3$) 9 (1H), 8·1 (2H, m), 7·8 (1H), 7·4 (3H, m), 7 (1H), 6·9 (1H), and 3·9 (6H) p.p.m., which was crystallised from petroleum (b.p. 80—100°) to give elongated prisms, m.p. 131—132° (Found: C, 76·8; H, 5·8; N, 5·4. $C_{17}H_{15}NO_2$ requires C, 77·0; H, 5·7; N, 5·3%).

The *methiodide hemihydrate*, obtained by use of dimethyl sulphate followed by ion exchange in potassium iodide solution, had m.p. 219—225° (decomp.) (from ethanol), $\nu_{max.}$ 1635 and 1615 cm^{-1}, δ [$(CD_3)_2SO$] 9·9 (1H), 8·3 (1H), 7·8 (7H, m), 4·2 (3H), and 4·05 (6H, d) p.p.m. (Found: C, 52·1; H, 4·45; N, 3·0. $C_{18}H_{18}INO_2,0{\cdot}5H_2O$ requires C, 51·9; H, 4·6; N, 3·4%).

2-Benzylamino-2-phenylacetamide.— Benzylidenebenzylamine (49 g) was treated with an excess of concentrated sodium disulphite solution, giving a slurry which was washed with ether. Excess of concentrated potassium cyanide solution was added, giving a clear solution with an oil, which was extracted with ether. Drying and evaporation gave 2-benzylamino-2-phenylacetonitrile (1b) (51 g, 91%). A solution of the aminonitrile (2·2 g) in concentrated sulphuric acid (20 ml) was heated on a boiling water bath for 3 h, cooled, and added carefully to water (200 ml); the resulting solution was washed with ether, basified with sodium hydrogen carbonate, and extracted with ether. Drying and evaporation of the extract gave the *aminoacetamide* as a white powder (1·34 g, 56%), which crystallised from aqueous ethanol as leaflets, m.p. 106—107°, and was recrystallised from ethyl acetate–petroleum (b.p. 80—100°) to give white rosettes, m.p. 116—117°, $\nu_{max.}$ 1690 and 3300 cm^{-1}, δ($CDCl_3$) 7·0 (10H, d), 6·35br (2H, d, exchangeable), 4·0 (1H), 3·6 (2H), and 2·0 (1H, exchangeable) p.p.m. (Found: C, 74·9; H, 6·7; N, 11·4. $C_{15}H_{16}N_2O$ requires C, 75·0; H, 6·7; N, 11·7%).

2-(N-Benzylmethylamino)-2-phenylacetamide.— 2-(*N*-benzylmethylamino)-2-phenylacetonitrile (1c) was prepared by the general method, from *N*-methylbenzylamine in place of 3,4-dimethoxybenzylamine, in 88% yield; it crystallised slowly, m.p. 45—47°, $\nu_{max.}$ 2230 cm^{-1}. The amino-nitrile (5 g) was treated as if for cyclisation with concentrated sulphuric acid; work-up in the usual way gave the *aminoacetamide* as a white solid, m.p. 124° (2·95 g, 55%), which was recrystallised from aqueous ethanol; m.p. 135°, $\nu_{max.}$ 1670, 3450, and 3200 cm^{-1}, δ ($CDCl_3$) 7·1 (10H, d), 6·5br (2H, d, exchangeable), 3·9 (1H), 3·35 (2H, d), and 2·0 (3H) p.p.m. (Found: C, 75·5; H, 6·9; N, 11·2. $C_{16}H_{18}N_2O$ requires C, 75·6; H, 7·1; N, 11·0%).

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Rearrangement in the Synthesis of Isoquinolines from Benzylaminoacetonitriles

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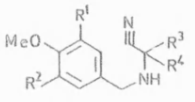
Summary The treatment of methoxybenzylaminoacetonitriles with concentrated sulphuric acid at 50° gave products arising from Hayashi-type rearrangement.

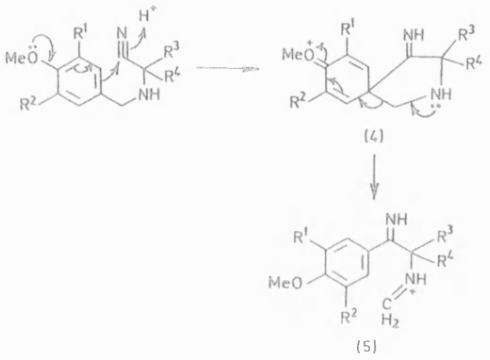
We have reported[1] the formation of 1,2-dihydro-6,7-dimethoxyisoquinolin-4(3*H*)-ones by treatment of 3,4-dimethoxybenzylaminoacetonitriles with concentrated sul-

phuric acid at 50°. Work described here suggests that contrary to generally accepted mechanisms for the Pomeranz–Fritsch synthesis[2] and its modifications, cyclisation in this case occurs preferentially but not exclusively *via* a



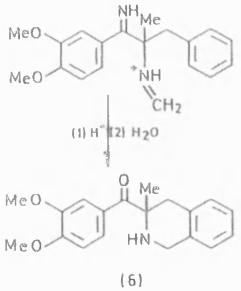
	R^1	R^2	R^3	R^4
(1)	H	OMe	Me	CH_2Ph
(2)	H	H	$-[CH_2]_5-$	
(3)	OMe	OMe	$-[CH_2]_5-$	

spiro-intermediate obtained from electrophilic attack *para* to the C-4 methoxy-substituent (Scheme 1).



SCHEME 1.

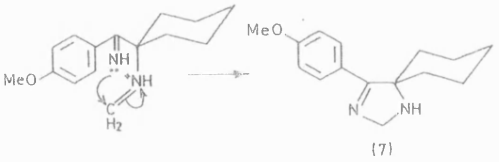
In the attempted preparation of a 3-benzylisoquinolinone from the aminonitrile (1), the sole product isolated was the isoquinoline (6), m.p. 138° (20% yield). We postulate a mechanism (Schemes 1 and 2) involving the spiro-intermediate (4), reminiscent of that suggested in the Hayashi rearrangement of *ortho*-benzoylbenzoic acids,[3] and subsequent formation of the iminium ion (5).



SCHEME 2.

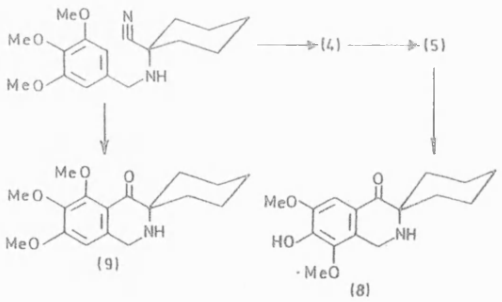
The fate of this intermediate is dependent upon the nature of the substituents R^3 and R^4 and the reactivity of the methoxy-substituted ring. The deactivating influence of the imino-group on the methoxy-ring favours Pictet–Spengler cyclisation to the benzyl substituent (5; R^4 = CH_2Ph) thus forming the 3,3-disubstituted-tetrahydroisoquinoline (Scheme 2).

The 4-methoxybenzylaminoacetonitrile (2) was converted into the spirocyclohexyl-3-imidazoline (7), m.p. 92° (80%) (Scheme 3). Here the low order of reactivity of the aromatic nucleus in the *ortho* positions and the absence of an alternative nucleophile allow only addition of the imine to the iminium ion with formation of the imidazoline ring.



SCHEME 3.

The hypothesis is supported by cyclisation of (3) (Scheme 4). Cyclisation *via* (5) to the spirocyclohexylisoquinolin-4(3*H*)-one (8), m.p. 149° occurred in 20—30% yield. The concomitant *O*-demethylation here observed is not in accord with the work of Grethe and his co-workers,[5] but may occur in the transition state (4), giving electronic stability and relieving steric strain. Such a central methyl group is comparatively easily lost under acidic conditions.[5] A small quantity (9%) of the "normal" spirocyclohexylisoquinolin-4(3*H*)-one (9), m.p. 128°, was also isolated. Thus "normal" cyclisation is not excluded.



SCHEME 4.

Preference for formation of the spiro-intermediate over electrophilic attack *para* to a C-3 methoxy-substituent is also evident in the cyclisation of 2-(2,3-dimethoxybenzylamino)-2-spirocyclohexylacetonitrile, where the only product isolated was 1,2-dihydro-5,6-dimethoxy-3-spirocyclohexylisoquinolin-4(3*H*)-one, m.p. 110°, in 9% yield.

Where the starting material possesses 3,4-dimethoxy-substitution and lacks alternative nucleophiles, as in the first examples we reported,[1] the product is the same whether produced by "normal" cyclisation or by rearrangement with cyclisation.

Structural assignments are based upon satisfactory elemental analysis, diagnostic i.r., n.m.r., and mass spectra

We thank Dr. A. M. Comrie for helpful discussions.

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[1] D. N. Harcourt and R. D. Waigh, *J. Chem. Soc.* (*C*), 1971, 967.
[2] W. J. Gensler, *Org. Reactions*, 1951, **6**, 191.
[3] R. B. Sandin, R. Melby, R. Crawford, and D. McGreer, *J. Amer. Chem. Soc.*, 1956, **78**, 3817; M. S. Newman and K. G. Ihrman, *ibid.*, 1958, **80**, 3652; S. J. Cristol and M. L. Caspar, *J. Org. Chem.*, 1968, **33**, 2020.
[4] G. Grethe, V. Toome, H. L. Lee, M. Uskokovic, and A. Brossi, *J. Org. Chem.*, 1968, **33**, 504.
[5] *E.g.*, A. Brossi, T. Van Burik, and S. Teitel, *Helv. Chim. Acta*, 1968, **51**, 1965.

Cyclisation of *N*-(Prop-2-ynyl)benzylamines. Part II.[1] Synthesis of 1,2-Dihydro-3-phenylisoquinolines and an Isopavine Derivative

By J. Robin Brooks, David N. Harcourt, and Roger D. Waigh,*† School of Pharmacy, Bath University, Claverton Down, Bath BA2 7AY

N-(1-Phenylprop-2-ynyl)- and *N*-methyl-*N*-(1-phenylprop-2-ynyl)-benzylamine cyclised in polyphosphoric acid to give good yields of 1,2-dihydro-4-methyl-3-phenylisoquinolines (2). The initial products of cyclisation were unstable and underwent atmospheric oxidation. The tertiary base (2; R = Me) gave an isoquinolin-1(2*H*)-one (7) and the secondary base (2; R = H) an unusual 1,4-dihydroisoquinolin-4-ol (8), which was readily dehydrated to the isoquinoline. Polyphosphoric acid treatment of *N*-(prop-2-ynyl)-1,2-diphenylethylamine (3) caused double cyclisation to give 10,11-dihydro-10,5-(iminomethano)-5-methyl-5*H*-dibenzo[*a*,*d*]cycloheptene (4) (an isopavine derivative).

We have reported the cyclisation of simple *N*-(prop-2-ynyl)benzylamines with polyphosphoric acid,[1] in which difficulties were encountered owing to the tendency of the primary reaction products to oxidise and disproportionate. We envisaged two ways in which the reaction could be investigated while avoiding these difficulties. In the first instance, we could take advantage of the ease of synthesis of 1-substituted propynols [2] to produce substituted bromo- and hence amino-propynes. Commencing with an aromatic aldehyde the resultant aminopropynes would cyclise to 3-aryl-1,2-dihydroisoquinolines (Scheme 1) which are reputedly stable [3] and are potentially adaptable intermediates in alkaloid synthesis.[4] Here the substituent chosen was phenyl, both for simplicity and because 1-phenylprop-2-yn-1-ol was commercially available.

In the second, a benzyl substituent attached to the

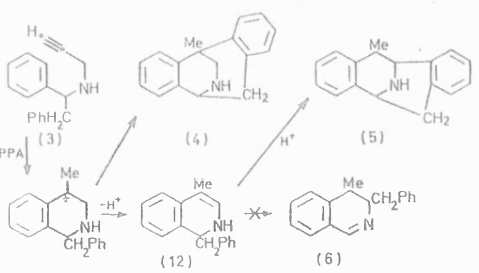
(1) (2)

Scheme 1

first-formed isoquinoline might trap the reactive intermediate in any of three ways [5-7] (Scheme 2).

Reaction of 1-phenylprop-2-yn-1-ol with phosphorus tribromide gave a good yield of the required 1-bromo-1-phenylprop-2-yne.[8] The bromo-compound was highly lachrymatory, like the nitrile analogue.[9] It was possible to distil it, despite a report [10] that the compound polymerised explosively on attempted isolation.

Alkylation of benzylamine and *N*-methylbenzylamine gave moderate yields of the required aminopropynes (1; R = H and R = Me). Cyclisation of the aminopropyne (1; R = Me) with polyphosphoric acid gave the expected 1,2-dihydro-4-methyl-3-phenylisoquinoline (2; R = Me)



Scheme 2

as an oil, identified by its n.m.r. spectrum. On exposure to the air for several days the oil solidified, and chromatography of the solid on alumina gave the isoquinolin-1(2*H*)-one (7). Similar reactions of 1,2-dihydroisoquinolines have been recorded,[11] and presumably a normal autoxidation occurs,[12] followed by dehydration of the peroxide (Scheme 3).

Cyclisation of the aminopropyne (1; R = H) also gave an oil, but in this case the n.m.r. spectrum could not be interpreted, the oil obviously consisting of a mixture of compounds. On trituration with an ether–petroleum mixture the oil solidified, and chromatography on alumina gave two crystalline materials. The first product showed i.r. absorption indicative of the presence of –OH and –C=N– groups. Elemental analysis and the presence in the n.m.r. spectrum of a two-proton AB quartet with geminal coupling (*J* 20 Hz) at δ 4·4 ($ArCH_2$–N=), together with other consistent absorption, suggested the 1,4-dihydroisoquinolin-4-ol structure (8).

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[1] J. R. Brooks and D. N. Harcourt, *J. Chem. Soc.* (*C*), 1969, 625, is regarded as Part I.

[2] *E.g.*, E. R. H. Jones and J. T. McCombie, *J. Chem. Soc.*, 1942, 733.

[3] S. F. Dyke and M. Sainsbury, *Tetrahedron*, 1966, **21**, 1907.

[4] D. N. Harcourt and R. D. Waigh, *J. Chem. Soc.* (*C*), 1971, 967.

[5] A. R. Battersby and D. A. Yeowell, *J. Chem. Soc.*, 1958, 1988.

[6] A. R. Battersby and R. Binks, *J. Chem. Soc.*, 1955, 2888.

[7] S. F. Dyke, *Adv. Heterocyclic Chem.*, 1972, **14**, 319.

[8] T. Y. Lai, *Bull. Soc. chim. France*, 1933, **53**, 1533.

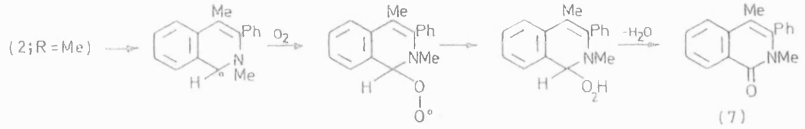
[9] J. P. Robinson, in 'Chemical and Biological Warfare,' ed. S. Rose, G. G. Harrap & Co. Ltd., London, 1968.

[10] M. Gaudemar, *Ann. Chim.* (*France*), 1956, **1**, 161.

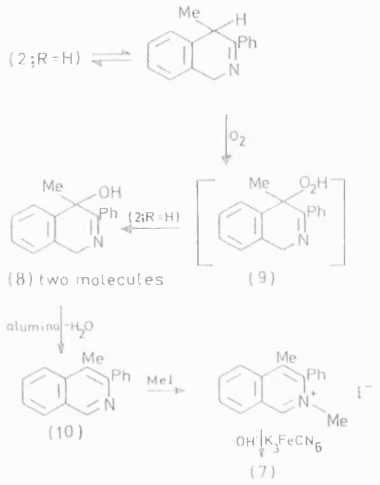
[11] M. Sainsbury, S. F. Dyke, and A. R. Marshall, *Tetrahedron*, 1966, **22**, 2447; *cf.* E. Hoeft, A. Rieche and H. Schultzr, *Annalen*, 1966, **697**, 181.

[12] A. G. Davies, 'Organic Peroxides,' Butterworths, London, 1961, pp. 28—30.

At first the formation of such a product was difficult to rationalise, but by analogy with recent work with other cyclic enamines,[13] it would appear that oxidation to the peroxide (9) may occur, which then ' proportionates ' with a molecule of the 1,2-dihydroisoquinoline (2; R = H) or its tautomer, to give two molecules of the dihydroisoquinolinol [(8), Scheme 4]. It seems that the imino-form of the dihydroisoquinoline (2; R = H) is subject to rapid oxidation, which would explain the difference in oxidation products of the secondary and tertiary bases, since in the absence of acid the latter is unable to tautomerise. The existence of both tautomeric forms of the isoquinoline (2; R = H) may explain the complexity of the n.m.r. spectrum of the crude cyclisation product, compared with that of the tertiary base (2; R = Me).



SCHEME 3



SCHEME 4

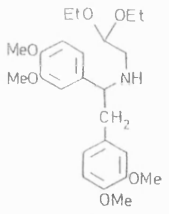
The second crystalline fraction from chromatography of the oxidised cyclisation product [from (1; R = H)] was identified as 3-methyl-3-phenylisoquinoline (10) (i.r., n.m.r., elemental analysis). The suspicion that this was an artefact from chromatography was confirmed on repassing a pure sample of the 1,4-dihydroisoquinolin-4-ol (8) through a similar alumina column, when quantitative conversion into isoquinoline (10) occurred (Scheme 4).

The isoquinoline (10) could be quaternised with methyl iodide, and the quaternary salt thus obtained oxidised with potassium ferricyanide in alkaline solution,[14] to give an isoquinolin-1(2*H*)-one (7), identical with that obtained from cyclisation of aminopropyne (1; R = Me).

No tendency to disproportionate [1] was found with any of the 3-phenylisoquinolines.

Cyclisation of *N*-(prop-2-ynyl)-1,2-diphenylethylamine (3) gave a crude yield (68%) of a viscous oil. The major component of the crude product was the ' isopavine ' derivative (4), for which the n.m.r. spectrum was diagnostic, although complicated by overlapping. Both AB and ABX spin coupling systems could be discerned owing to the non-equivalence of the protons of each $-CH_2-$ group, with large geminal coupling constants. Significantly, the methyl group signal was a sharp singlet, eliminating possible alternative structures (5) and (6). A mass spectrum and accurate mass measurement of the parent ion confirmed the structure of the major product.

Some impurities were indicated in the n.m.r. spectrum of the crude material, notably a sharp singlet at δ 2·1 of unknown origin, and a doublet at δ 1·46 which in theory could have originated from a variety of products, including (5) and (6). Further work by another group [15] interested in the pharmacological activity of the isopavine derivative (4) has indicated that the latter impurity was probably the pavine derivative (5).



It thus appears that the aminopropyne (3) cyclises in a very similar manner to the acetal (11) used by

[13] G. Berti, A. Da Settimo, G. Di Colo, and E. Nannipieri, *J. Chem. Soc.* (*C*), 1969, 2703.

[14] R. D. Haworth and W. H. Perkin, *J. Chem. Soc.*, 1925, 1434.

[15] D. C. Bishop, personal communication.

Battersby [5] to obtain 'isopavine,' and that the dihydroisoquinoline [(12), Scheme 2] is not formed to any great extent.

EXPERIMENTAL

Unless stated, m.p.s were taken with a Kofler hot-stage apparatus, and are corrected. I.r. spectra were obtained with a Unicam SP 200 instrument for potassium bromide discs or liquid films. N.m.r. spectra were recorded with a Varian A60 instrument (tetramethylsilane as internal reference).

1-Bromo-1-phenylprop-2-yne.—1-Phenylprop-2-ynol (100 g) and dry pyridine (5 g) were dissolved in dry ether (100 ml). Phosphorus tribromide (80 g) was added dropwise with stirring, and the mixture left overnight. The solution was refluxed for 2 h, cooled, poured into water (400 ml), and the ether layer decanted. The water layer was decanted from the heavy oil which had separated, and the oil added to the ether layer. The water layer was extracted with ether, and the ether layers were combined, washed with sodium hydrogen carbonate solution, dried, and evaporated. The residual oil was distilled under high vacuum, the distillation being stopped when the rate slowed appreciably (in one experiment the temperature was allowed to rise and the boiler residue decomposed vigorously, resulting in loss of the product; see text). The *bromide* (90·5 g, 61%) was a mobile, pale yellow liquid with strong lachrymatory properties, sufficiently pure for further reactions. The analytical sample was twice fractionally distilled, b.p. 76—80° at 2·5 mmHg, $\nu_{max.}$ 2120 and 3300 cm^{-1} (Found: C, 55·3; H, 3·6; Br, 40·7. C_9H_7Br requires C, 55·4; H, 3·6; Br, 41·0%).

N-(1-Phenylprop-2-ynyl)benzylamine (1; R = H).—Benzylamine (53·5 g, 0·5 mol), was dissolved in sodium-dried benzene (300 ml). 1-Bromo-1-phenylprop-2-yne (32·5 g, 0·167 mole), in sodium-dried benzene (150 ml), was added dropwise, with stirring. Stirring was continued 2 h, the solution refluxed 2 h, and allowed to stand 48 h. The benzene solution was shaken with 5N-sodium hydroxide solution (100 ml), and separated. The aqueous layer was extracted with benzene. After drying, evaporation of the combined benzene solutions gave an oil. Fractionation (at 1 mmHg) gave benzylamine (32 g) and then the *benzylamine* (1; R = H) (16 g, 43%). The analytical sample was redistilled, b.p. 136—137° at 0·7 mmHg, $\nu_{max.}$ 3300 cm^{-1}, δ (CCl_4) 7·85 (10H, m), 4·45 (1H, m), 3·75 (2H, m), and 0·45 (1H, exchangeable) (Found: C, 86·6; H, 6·8; N, 6·55. $C_{16}H_{15}N$ requires C, 86·9; H, 6·8; N, 6·3%).

N-Methyl-N-(1-phenylprop-2-ynyl)benzylamine (1; R = Me).—The procedure was initially as above, but the distillation was stopped after removal of *N*-methylbenzylamine, and the residue was cooled and extracted with ether. The ether solution was extracted with dilute hydrochloric acid, this solution basified, and re-extracted with ether. Drying and removal of solvent gave the *benzylamine* (1; R = Me) (14·9 g, 38%), as a pale brown oil which crystallised on cooling at 0° for 7 days (m.p. 34—41°). Recrystallisation from aqueous ethanol gave prisms, m.p. 43—45°, $\nu_{max.}$ 3300 cm^{-1}, δ (CCl_4) 7·1 (10H, m), 4·5 (1H, m), 3·45 (2H), 2·35 (1H, m), and 2·05 (3H) (Found: C, 86·9; H, 7·15; N, 6·0. $C_{17}H_{17}N$ requires C, 86·8; H, 7·3; N, 5·95%).

1,4-Dihydro-4-methyl-3-phenylisoquinolin-4-ol (8) *and 4-Methyl-3-phenylisoquinoline* (10).—The aminopropyne (1; R = H) (2·18 g) was heated with polyphosphoric acid (20 g) for 6 h at 140° with stirring. The mixture was cooled, diluted with ice–water, made alkaline with strong ammonium hydroxide solution with addition of ice, and extracted with ether. Drying and evaporation gave a viscous, pale brown oil (1·78 g, 82%), which showed no –OH absorption in the i.r. spectrum. Trituration with ether–petroleum (b.p. 40—60°) caused solidification to a brown granular material, m.p. *ca.* 130°, with strong –OH absorption, $\nu_{max.}$ 3180 cm^{-1}. Chromatography with benzene on an alumina column (B.D.H. alumina, untreated), gave the *dihydroisoquinolinol* (8), a pale yellow, crystalline solid (0·92 g), which was recrystallised from benzene–petroleum (b.p. 40—60°) as needles, m.p. 139—141°, $\nu_{max.}$ 3180 and 1625 cm^{-1}, δ 7·1 (9H, m), 3·95—4·85 (2H, ABq, *J* 20 Hz), 3·35 (1H, exchangeable), and 1·35 (3H) (Found: C, 80·85; H, 6·4; N, 5·9. $C_{16}H_{16}NO$ requires C, 81·0; H, 6·3; N, 5·9%).

Continued elution with benzene gave *4-methyl-3-phenylisoquinoline* (10) as a crystalline solid (0·305 g), which was recrystallised from petroleum (b.p. 40—60°), as prisms, m.p. 103—104°, $\nu_{max.}$ 1620 cm^{-1}, δ 9·2 (1H), 7·7 (9H, m), and 2·6 (2H) (Found: C, 87·7; H, 6·0; N, 6·25. $C_{16}H_{13}N$ requires C, 87·7; H, 5·9; N, 6·4%).

When the chromatography was carried out using petroleum (b.p. 40—60°) initially, with increasing proportions of benzene, the material remained in contact with the alumina for a longer time, and only 4-methyl-3-phenylisoquinoline was isolated (65% yield based on aminopropyne). Similarly, pure isoquinolinol (8) gave a quantitative yield of the aromatic isoquinoline (10) on passing through an alumina column eluted first with petroleum (b.p. 40—60°), then 10, 20, 50%, and finally pure benzene.

2,4-Dimethyl-3-phenylisoquinolinium Iodide.—The *salt* formed very slowly as long yellow needles when excess of methyl iodide was added to a dry ethereal solution of the isoquinoline (10), m.p. 263—265° (decomp., capillary tube) (Found: C, 56·1; H, 4·65; N, 3·9. $C_{17}H_{16}IN$ requires C, 56·5; H, 4·5; N, 3·9%).

2,4-Dimethyl-3-phenylisoquinolin-1(2H)-one (7).—(a) *From 2,4-dimethyl-3-phenylisoquinolinium iodide.* The isoquinolinium methiodide (see above, 0·47 g) was dissolved in aqueous ethanol (10 ml). A solution of potassium hydroxide (0·5 g) and potassium hexacyanoferrate(III) (1·4 g) in water (10 ml) was added, giving a white precipitate which dissolved on warming. The solution was cooled and extracted with ether, which on evaporation gave a pale yellow solid (0·285 g, 88%), m.p. and mixed m.p. with the isoquinolin-1(2*H*)-one obtained below, 104—105° and having an identical i.r. spectrum.

(b) *From N-methyl-N-(1-phenylprop-2-ynyl)benzylamine* (1; R = Me). The aminopropyne (1; R = Me) (2·32 g), treated as described for cyclisation of aminopropyne (1; R = H) above, gave an oil (2·05 g, 88%). By evaporating solvents under nitrogen and working quickly, the primary product, 1,2-dihydro-2,4-dimethyl-3-phenylisoquinoline, could be obtained in a high state of purity, as determined by the n.m.r. spectrum; δ 7·1 (9H, m), 4·2 (2H), 2·35 (3H), and 1·85 (3H). After several days' exposure to air the oil solidified, and chromatography with petroleum (b.p. 40—60°) on an alumina column gave the *isoquinolinone* (7) (61% based on aminopropyne), as white needles, m.p. 107—108° [from petroleum (b.p. 80—100°)], $\nu_{max.}$ 1640 cm^{-1}, δ 8·2 (1H, m), 7·15 (8H, m), 3·1 (3H), and 1·9 (3H) (Found: C, 81·7; H, 6·0; N, 5·6. $C_{17}H_{16}NO$ requires C, 81·9; H, 6·1; N, 5·6%).

N-(Prop-2-ynyl)-1,2-diphenylethylamine (3) *Hydrochloride*.—3-Bromopropyne (3·0 g) in dry ether (50 ml) was added dropwise to a solution of 1,2-diphenylethylamine (15 g) in ether (20 ml). After 48 h the crystals were filtered off and the solution extracted with dilute hydrochloric acid. A solid (4·3 g) crystallised from the acid solution and was filtered off. Recrystallisation from ethanol–acetone gave the *hydrochloride* as needles, m.p. 198—200° (2·8 g, 60%), δ (free base, CCl_4) 6·9 (10H, m), 3·95 (1H, t, *J* 7 Hz), 2·95 (2H, d, *J* 3 Hz), 2·7 (2H, d, *J* 7 Hz), 1·9 (1H, t, *J* 3 Hz), and 1·35 (1H, exchanged with D_2O) (Found: C, 74·8; H, 6·55; N, 4·8. $C_{17}H_{16}ClN$ requires C, 75·1; H, 6·6; N, 5·2%).

10,11-Dihydro-5-methyl-10,5-(iminomethano)-5H-dibenzo-[a,d]cycloheptene (4).—*N*-(Prop-2-ynyl)-1,2-diphenylethylamine hydrochloride (2·2 g) was dissolved in polyphosphoric acid (20 g) and heated at 145° for 6 h. The solution was diluted with water (100 ml) and made alkaline after being left for 30 min. The n.m.r. spectrum of the oil was obtained by the Physico-Chemical Measurements Unit, Harwell, at 100 MHz in $CDCl_3$ solution, δ 7·1 (8H, m), 4·2 (1H, t, *J* apparent 3·5 Hz), 3·2 (4H, AB or ABX, J_{AX} apparent = J_{BX} apparent = 3·5, J_{AB} 17 and AB, *J* 12 Hz), 2·0 (1H, exchangeable), and 1·8 (3H). The base, regenerated from the hydrochloride, showed a single peak on g.l.c. using 2½% SE 301 silicone gum rubber on Chromosorb G. The *hydrochloride* was used to obtain the mass spectrum (Found: M^+, 235·1360. $C_{17}H_{17}N$ requires *M*, 235·1361).

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Synthesis of 3-Arylisoquinolines by Thermolysis of 3-Aryl-1,2-dihydroisoquinolin-4(3*H*)-one Salts

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Summary 3-Aryl-1,2-dihydroisoquinolin-4(3*H*)-one salts give mixtures of 3-arylisoquinolines and 3-aryl-4-hydroxyisoquinolines after heating in dimethylformamide and extraction.

MASS spectra of salts (hydrochlorides or hydrogen sulphates) of 3-aryl-1,2-dihydroisoquinolin-4(3*H*)-ones (1) show two main peaks, $M - 2H$ and $M - H_2O$. The former corresponds to loss of 2H from the 1,2-bond with enolisation to give the aromatic 4-hydroxyisoquinoline (2) and the latter suggests loss of oxygen attached to the 4-position and two hydrogens to give the 4-unsubstituted aromatic isoquinoline (3). The first process is expected, but the loss of doubly-bonded oxygen is difficult to explain by the usual fragmentation pathways, since it involves a dual reduction–oxidation occurring simultaneously at different parts of the heterocycle. We therefore envisaged a thermal process, and repeated it by heating the salts in evacuated tubes. This was successful with very small samples, but with synthetically useful quantities gums were produced from which only small amounts of product could be isolated. We resorted to the use of a high-boiling inert solvent

[dimethylformamide (DMF)], and obtained a satisfactory synthetic procedure, which replaces the three steps previously necessary to effect the conversion.[1] Yields of (2) and (3), after work-up and isolation by preparative thin-layer or column chromatography in four cases, are given in the Table.

Table

R	Anion	Yield (%) (3)	Yield (%) (2)
Ph	HSO_4^-	56	14
		64[a]	12[a]
3,4-Dichlorophenyl	HSO_4^-	43	29
4-Pyridyl	Cl^-	23[b]	44
3,4-Dimethoxyphenyl	Cl^-	25	38
		52[a]	21[a]
2-Pyridyl		see text	
H	Cl^-	see text	

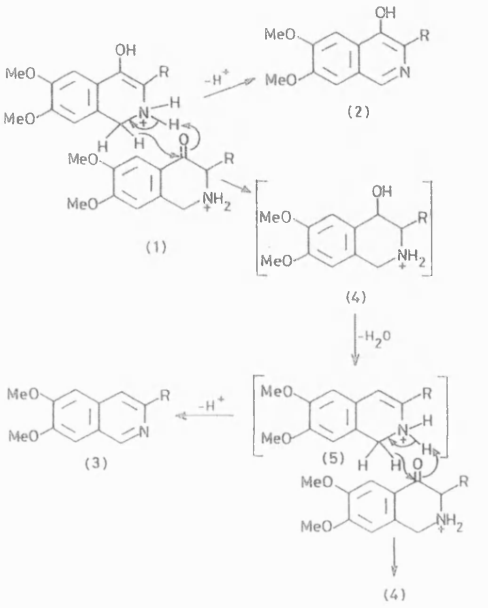
[a] Under nitrogen. [b] Also some *O*-demethylated isoquinoline (9%).

Since the 4-hydroxyisoquinoline (2) is an expected product of air oxidation we repeated two of the reactions under nitrogen and, as expected, the yield of (3) was increased (see Table).

While this thermolysis is reminiscent of the synthesis of naphthalenes from 3,4-dihydronaphthalen-1(2*H*)-ones[2,3] and of phenols from cyclohexane-1,4-diones,[4] the reaction conditions are different. Naphthalene production is strongly hindered by a substituent adjacent to the carbonyl,[3] which also suggests a different mechanism. Since the 4-hydroxyisoquinoline (2) is produced under nitrogen and in high vacuum, we suggest an intermolecular mechanism (Scheme), where the hydroxytetrahydroisoquinoline (4) is re-generated. Dehydration of (4) would give the powerfully reducing[5] 1,2-dihydroisoquinoline (5). Thus every time the chain was initiated, a molecule of (2) would be produced, but thereafter consumption of (1) would produce only (3).

Alternative intramolecular and bimolecular (head-to-tail) mechanisms fail to account for the production of (2). However, it must be recognised that oxidation to the 4-hydroxyisoquinoline is very easy. In the case where R = 2-pyridyl, we have been unable to isolate the isoquinolinone from the previous synthetic step, the product oxidising rapidly to (2; R = 2-pyridyl).

Mass spectra of the free bases of these 3-arylisoquinolinones also exhibit peaks corresponding to $M - 2H$ and $M - H_2O$, but these no longer represent the major fragments. The main peaks correspond instead to a *retro*-Diels–Alder homolysis in which the nitrogen atom is lost together with C-3 and the 3-substituent as a benzylidene imine. Where the 3-substituent is lacking as in (1; R = H), this mode of decomposition leads to loss of methylene imine even when the hydrochloride is used, and there is no sign of aromatisation to isoquinoline (3). The hydrochloride (1; R = H) also gave an intractable product when heated in DMF, as did the free base when fused with NaOH–KOH.[2] The major function of the 3-aryl substituent in the thermolysis is probably to assist enolisation (Scheme).



Scheme

We thank the University of Strathclyde for a scholarship (to D. A. L.).

(*Received*, 3*rd July* 1978; *Com.* 695.)

[1] D. N. Harcourt and R. D. Waigh, *J. Chem. Soc.* (*C*), 1971, 967.
[2] A. J. Birch and D. A. White, *J. Chem. Soc.*, 1964, 4086.
[3] J. M. Springer, C. W. Hinman, E. J. Eisenbraun, P. W. K. Flanagan, and M. C. Hanning, *J. Org. Chem.*, 1970, **35**, 1260.
[4] C. G. Rao, S. Rengaraju, and M. V. Bhatt, *J.C.S. Chem. Comm.*, 1974, 584.
[5] H. Schmid and P. Karrer, *Helv. Chim. Acta*, 1949, **32**, 960.

Cyclisation of Benzylaminoacetonitriles. Part 2.[1] Evidence for Two Mechanisms of Cyclisation

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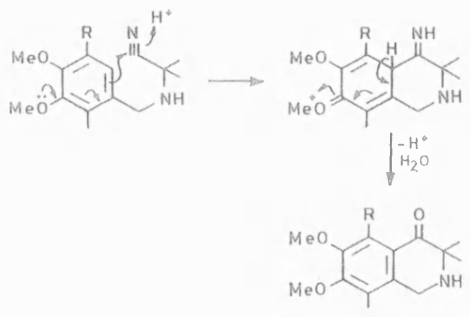
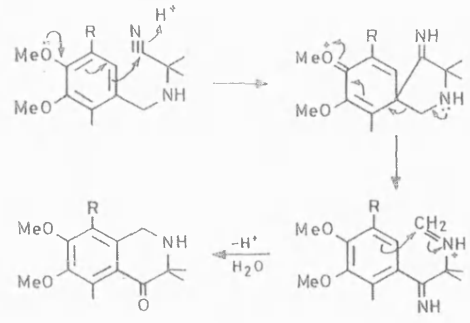
Treatment of 3,4-dimethoxybenzylaminoacetonitriles with concentrated sulphuric acid gives 2,3-dihydroisoquinolin-4(1*H*)-ones. Cyclisation of 1-(3,4,5-trimethoxybenzylamino)cyclohexanecarbonitrile (2) at room temperature gave 3,4-dihydro-5,6,7-trimethoxyisoquinoline-3-spirocyclohexan-4(1*H*)-one (8) (22%) and 1-(3,4,5-trimethoxybenzylamino)cyclohexanecarboxamide (9) (7%). At 50 °C, cyclisation gave 3,4-dihydro-7-hydroxy-6,8-dimethoxyisoquinoline-3-spirocyclohexan-4(1*H*)-one (10) as the major product. A dual mechanism for cyclisation is postulated, one mode involving electrophilic attack *para* to the C-3 methoxy-substituent, the second attack *para* to the C-4 methoxy-substituent, giving a spiro-intermediate which undergoes rearrangement to an iminium ion, the fate of which is dependent on the proximity of differing nucleophiles. The latter mechanism is consistent with the formation of 1,2,3,4-tetrahydro-6,7-dimethoxy-2-(4-methoxyphenacyl)isoquinoline by treatment of 2-[*N*-(3,4-dimethoxyphenethyl)-4-methoxybenzylamino]acetonitrile (6) with sulphuric acid.

We have described [1] the synthesis of 2,3-dihydro-6,7-dimethoxyisoquinolin-4(1*H*)-ones from veratrylaminoacetonitriles, and have postulated a mechanism involving



Scheme 1

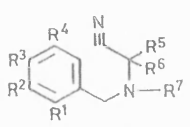
orthodox cyclisation *via* electrophilic attack *para* to the C-3 methoxy-substituent (Scheme 1). Subsequent work, described here, has indicated that cyclisation may



Scheme 2

proceed preferentially by attack *para* to the C-4 methoxy-group (Scheme 2).

The cyclisation of 3,4-dimethoxybenzylaminoacetonitriles [*e.g.* (1)], which lack alternative nucleophilic sites for attack by the iminium ion, would yield the same isoquinoline by orthodox cyclisation or *via* the spiro-intermediate (Schemes 1 and 2, R = H). The 3,4,5-trimethoxybenzylaminonitrile (2) would be expected to yield a 5,6,7-trimethoxyisoquinolinone according to Scheme 1 (R = OMe), but the 6,7,8-trimethoxyisoquinolinone by Scheme 2 (R = OMe). Cyclisation of the amino-nitrile (2) with concentrated sulphuric acid at room temperature gave, after dilution and basification with 5N-sodium hydroxide, an oil which on fractional crystallisation from petroleum gave the amide (9) in 7% yield and the 5,6,7-trimethoxyisoquinolone (8) in 22% yield (Scheme 3). The orientation of the methoxy-groups in 8 was unambiguously established by the chemical shift of the signal due to the aromatic proton, which appears as a singlet at δ 6.38. We envisage the amide (9) being formed by either hydration of the nitrile or hydrolytic attack on the spiro-intermediate (Scheme 3).



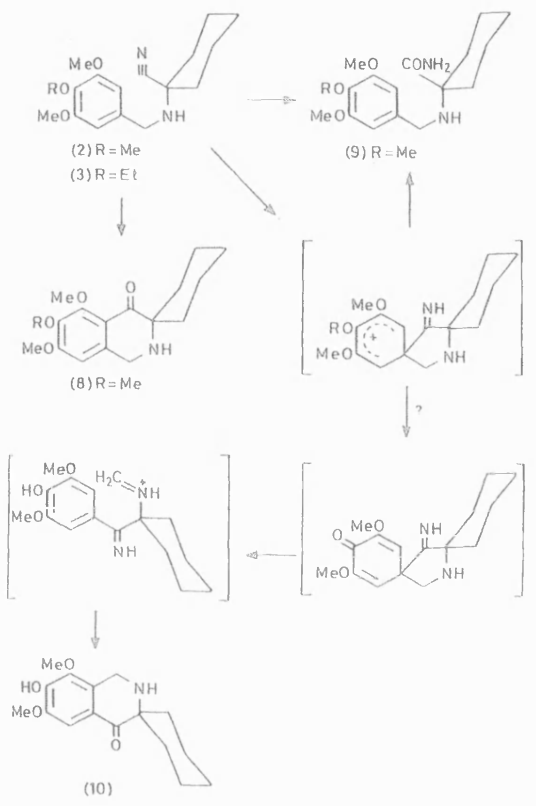
	R^1	R^2	R^3	R^4	R^5	R^6	R^7
(1) *	H	MeO	MeO	H	C_5H_{10}		H
(2)	H	MeO	MeO	MeO	C_5H_{10}		H
(3)	H	MeO	EtO	MeO	C_5H_{10}		H
(4)	H	MeO	H	H	C_5H_{10}		H
(5)	H	MeO	H	H	H	H	H
(6)	H	H	MeO	H	H	H	DMPE
(7)	MeO	MeO	H	H	C_5H_{10}		H

* Previously reported.[1] DMPE = 3,4-dimethoxyphenethyl.

These products were isolated in the same manner when cyclisation was carried out at 50 °C. The major product was obtained by addition of concentrated hydrochloric acid to the aqueous alkaline solution, which gave a

[1] Part 1, D. N. Harcourt and R. D. Waigh, *J. Chem. Soc.* (*C*), 1971, 967.

copious white precipitate from which was regenerated a phenolic isoquinolinone with sodium hydrogen carbonate solution. Recrystallisation from petroleum gave 2,3-dihydro-7-hydroxy-6,8-dimethoxyisoquinoline-3-spiro-cyclohexan-4(1*H*)-one (10) (Scheme 3) in 20% yield.



Scheme 3

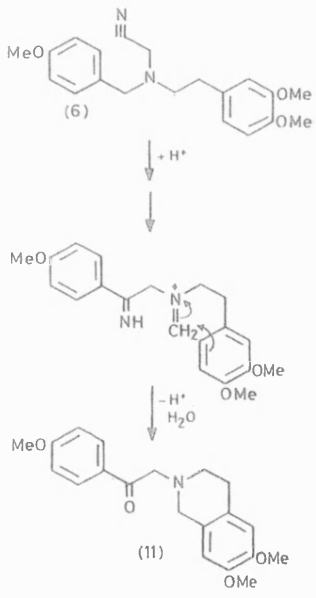
Here, orientation of the aromatic substituent groups was less readily established. Location of the aromatic proton at C-5 was unambiguous on the basis of n.m.r. data, the signal appearing at δ 7.34 owing to deshielding by the C-4 carbonyl function. Double irradiation experiments confirmed a C-6 methoxy-substituent, long-range coupling and the nuclear Overhauser effect being observed between the methoxy-group and the C-5 proton.

Orientation of the remaining methoxy-group could not be established by these techniques. Similarly, attempts to remove the phenolic hydroxy-group by the method of Lonsky and his co-workers [2] were also unsuccessful (dehydroxylation at C-8 would yield a product previously characterised,[1] and at C-7 the product should show *meta*-coupling with the C-5 proton in the n.m.r. spectrum).

Finally identification was accomplished by cyclisation of 1-(4-ethoxy-3,5-dimethoxybenzylamino)cyclohexane-carbonitrile (3), which gave the same phenolic isoquinolone (10).

Whilst the ' central ' methyl group is relatively easily removed by sulphuric acid, as for example in the preparation of syringic aldehyde, it is possible that demethylation occurs in the transition state to give a dienone which might then undergo rearrangement to the phenol (10). There is evidence from other amino-nitrile cyclisations [3] that the latter process may contribute, but in this case we are unable to exclude the possibility that demethylation occurs at a penultimate stage after rearrangement has been completed.

Further evidence for Scheme 2 being the favoured mechanism is seen in the cyclisation of 2-[*N*-(3,4-dimethoxyphenethyl)-4-methoxybenzylamino]acetonitrile (6), which gave 1,2,3,4-tetrahydro-6,7-dimethoxy-2-(4-methoxyphenacyl)isoquinoline (11) (Scheme 4), in 33% yield. This latter reaction clearly demonstrates the ability of the iminium ion to cyclise by interaction with an alternative and more reactive nucleophile when one is present. The structure was unequivocally confirmed by spectroscopy and by an unambiguous synthesis from 1,2,3,4-tetrahydro-6,7-dimethoxyisoquinoline and 4-methoxyphenacyl bromide.



Scheme 4

Benzylamino-nitriles with methoxy-substituents in the 2- and 3- or the 3-position [(4), (5), and (7)] and with C-4 unsubstituted were extensively sulphonated during

[2] W. Lonsky, H. Traitler, and K. Kratyl, *J.C.S. Perkin I*, 1975, 169.
[3] R. D. Waigh, unpublished work.

the attempted cyclisation. The 2,3-dimethoxy compound (7) gave 2,3-dihydro-5,6-dimethoxyisoquinoline-3-spirocyclohexan-4(1*H*)-one in 5% yield, the main product being an uncharacterised sulphonic acid. The orientation of the methoxy-groups in this isoquinolinone was assigned on n.m.r. evidence. The calculated chemical shifts of the two aromatic protons (δ 6.90 and δ 7.05) are in agreement with the experimental values, whereas the chemical shifts of the aromatic protons in the 7,8-dimethoxy-isomer are calculated as δ 6.84 and 7.45. Cyclisation must therefore proceed *via* the spiro-intermediate, the activating group being the C-2 methoxy-group.

The cyclisation of benzylaminonitriles with only a *meta*-methoxy-substituent necessarily follows Scheme 1, 3-methoxybenzylaminoacetonitrile (5) giving the known 2,3-dihydro-7-methoxyisoquinolin-4(1*H*)-one in 27% yield. The spirocyclohexane analogue (4) however, did not cyclise, the only product isolated being an uncharacterised sulphonic acid. Elemental and spectroscopic analysis showed this to be a 1-(3-methoxybenzylamino)cyclohexanecarboxamide sulphonic acid, but the orientation of the acid group could not be determined owing to the complexity of the aromatic n.m.r. signal.

EXPERIMENTAL

M.p.s were taken on a Kofler hot-stage apparatus and are corrected. I.r. spectra were obtained with a Unicam SP 200 instrument. N.m.r. spectra were determined for solutions in deuteriochloroform with JEOL P5100 and Varian HA-100 instruments. Mass spectral data were obtained with an A.E.I. MS 12 instrument in the School of Chemistry, University of Bath.

The benzylaminoacetonitriles (1)—(7) were obtained as reported.[1]

Cyclisation of Benzylaminoacetonitriles. General Method.—The benzylaminoacetonitrile (5 g) was added carefully to concentrated sulphuric acid (25 ml) in an ice-bath, with stirring continuously until dissolution was complete. The solution was kept at room temperature overnight (' cold ' cyclisation) or heated to 50 °C for 4 h (' hot ' cyclisation), diluted by pouring on to crushed ice, and set aside for 30 min. The diluted mixture was basified with aqueous sodium hydroxide (20% w/v), ice being added from time to time to prevent an excessive rise in temperature. The product was either filtered off or extracted with chloroform, washed, dried, and recrystallised from petroleum (b.p. 60—80 °C).

Cyclisation of 1-(3,4,5-*trimethoxybenzylamino*)*cyclohexanecarbonitrile* (2). *' Cold ' cyclisation.* The chloroform extract gave on evaporation an oil (1.6 g) which after refluxing with petroleum (b.p. 40—60 °C) gave 1-(3,4,5-*trimethoxybenzylamino*)*cyclohexanecarboxamide* (9) (375 mg, 7%), m.p. 154°, as small white prisms, $\nu_{max.}$ 3 200 and 3 450 (NH_2 amide), 3 320 (NH amine), and 1 660 cm^{-1} (CO); δ 7.17br (1 H, s, exch. NH amide), 6.56 (2 H, s, ArH), 6.14br (1 H, s, exch. NH amide), 3.84—3.88 (9 H, 2 s, 3 MeO), 3.56 (2 H, s, $ArCH_2$), 1.6 (11 H, m, C_5H_{10} and exch. NH); *m/e* 322 (M^+, <1%), 278 (90), 196 (50), and 181 (100) (Found: C, 63.7; H, 8.1; N, 8.5. $C_{17}H_{26}N_2O_4$ requires C, 63.4; H, 8.1; N, 8.7%). Concentration of the petroleum solution yielded 2,3-*dihydro*-5,6,7-*trimethoxyisoquinoline*-3-*spirocyclohexan*-4(1H)-*one* (8) (1.1 g, 22%), m.p. 128°; $\nu_{max.}$ 3 290 (NH) and 1 650 cm^{-1} (CO); δ 6.38 (1 H, s, ArH), 4.00 (2 H, s, $ArCH_2$), 3.90 (6 H, s, 2MeO), 3.84 (3 H, s, MeO), 1.85 (1 H, s, exch. NH), and 1.60 (10 H, m, C_5H_{10}); *m/e* 305 (M^+, 31%), 303 (10), 288 (5), 277 (3), 234 (3), 208 (100), 193 (20), 181 (72), and 165 (20%) (Found: C, 66.5; H, 7.2; N, 4.6. $C_{17}H_{23}NO_4$ requires C, 66.8; H, 7.5; N, 4.6%).

The extracted aqueous solution was reacidified with concentrated hydrochloric acid, basified with potassium hydrogen carbonate, and extracted with chloroform. Drying ($MgSO_4$) and removal of the chloroform gave a small amount of unidentified intractable gum.

TABLE 1

Spectroscopic data for benzylaminoacetonitriles

Compd.	$\nu_{max.}$/cm^{-1} NH	C≡N	δ_H
(2)	3 300	2 200	6.70 (2 H, s, ArH), 3.85 (11 H, d, 3 MeO and $ArCH_2$), 1.0—2.2 (11 H *, m, C_5H_{10} and NH)
(3)	3 300	2 220	6.60 (2 H, s, ArH), 4.05 (2 H, q, *J* 7 Hz, CH_3CH_2O), 3.85 (8 H, s, 2 MeO and $ArCH_2$), 1.7 (11 H *, m, C_5H_{10} and NH), 1.35 (3 H, t, *J* 7 Hz, CH_3CH_2)
(4)	3 300	2 240	6.6—7.4 (4 H, m, ArH), 3.85 (2 H, s, $ArCH_2$), 3.75 (3 H, s, MeO), 1.2—2.2 (11 H *, m, C_5H_{10} and NH)
(5)	3 370	2 200	6.6—7.4 (4 H, m, ArH), 3.50 (2 H, s, $ArCH_2$), 3.40 (2 H, s, NCH_2CN), 1.80 (1 H *, s, NH)
(6)		2 230	7.20 (2 H, d, *J* 9 Hz, MeO C_6H_4), 6.85 (2 H, d, *J* 9 Hz, MeO C_6H_4), 6.71 (3 H, m, $(MeO)_2C_6H_3$), 3.70 (6 H, s, 2 MeO), 3.6 (3 H, s, MeO), 3.62 (2 H, s, $ArCH_2N$), 3.43 (2 H, s, NCH_2CN), 2.80 (4 H, s, N-$[CH_2]_2$Ar)
(7)	3 350	2 250	6.9 (3 H, m, ArH), 3.82 (6 H, s, 2 MeO), 3.42 (2 H, s, $ArCH_2$), 2.50 (1 H *, s, NH), 1.85 (10 H, m, C_5H_{10})

* Integral reduced by 1 H after deuteriation.

' Hot ' cyclisation. The dried chloroform extract, on evaporation and fractional crystallisation from petroleum (b.p. 40—60 °C) gave the amide (9) (320 mg, 6%), m.p. 154°, mixed m.p. 154°, and the isoquinolone (8) (360 mg, 6%), m.p. 128°, mixed m.p. 128°.

TABLE 2

Benzylaminoacetonitriles

Compd.	Yield (%)	M.p.(°C)	Found(%) C	H	N	Required(%) C	H	N
(2)	79	70	67.2	7.9	9.1	67.1	7.9	9.2
(3)	89	74	68.3	8.3	8.8	67.9	8.2	8.8
(4)	84	oil	74.0	8.3	11.4	73.8	8.2	11.5
(5)	61	129 (decomp.) *	56.5	6.2	13.3	56.6	6.2	13.2
(6)	63	62	71.4	7.2	8.2	70.6	7.1	8.2
(7)	42	50	70.0	7.8	10.3	70.0	8.1	10.2

* Base HCl.

Reacidification of the extracted aqueous solution with an excess of concentrated hydrochloric acid gave a copious white precipitate (1.4 g) which was collected and suspended in water (100 ml). An excess of potassium hydrogen carbonate was added, the mixture was extracted three times with chloroform (40 ml), and the combined extracts were washed once with water (10 ml), dried ($MgSO_4$), and evaporated. Recrystallisation of the residue from petroleum (b.p. 60—80 °C) gave 2,3-*dihydro*-7-*hydroxy*-6,8-

dimethoxyisoquinoline-3-spirocyclohexan-4(1H)-one (10) (1.0 g, 20%), m.p. 149°; $\nu_{max.}$ 3 450 (OH), 3 350 (NH), and 1 660 cm^{-1} (CO); δ 7.34 (1 H, s, ArH), 4.06 (4 H, s with shoulder, $ArCH_2$, exch. NH and OH), 3.90 (3 H, s, MeO), 3.88 (3 H, s, MeO), and 1.6 (10 H, m, C_5H_{10}); *m/e* 291 (M^+, 40%), 290 (5), 263 (4), 248 (4), 232 (40), 220 (20), 194 (18), 167 (100), and 145.5 (10) (Found: C, 66.2; H, 7.3; N, 4.7. $C_{16}H_{21}NO_4$ requires C, 66.0; H, 7.2; N, 4.8%).

Cyclisation of 1-(4-*ethoxy*-3,5-*dimethoxybenzylamino*)*cyclohexanecarbonitrile* (3). *'Hot' cyclisation.* After basification the mixture was washed with chloroform and the organic layer discarded. Reacidification with concentrated hydrochloric acid gave a white precipitate which was collected and worked up as described in the preparation of the isoquinolone (10). Crystallisation from petroleum (b.p. 60—80 °C) gave white crystals (1.5 g, 30%), m.p. 149°, which i.r. and n.m.r. spectra showed to be identical with the isoquinolinone (10) (mixed m.p. 149°).

Cyclisation of 2-(3-*methoxybenzylamino*)*acetonitrile* (5). *'Hot' cyclisation.* The chloroform extract gave 2,3-dihydro-7-methoxyisoquinolin-4(1*H*)-one as a white solid (1.07 g, 27%). The hydrochloride was prepared by addition of an excess of ethereal hydrogen chloride to an ether–propan-2-ol solution and after crystallisation from ether–methanol (1 : 1) had m.p. 216—218° (decomp.) (capillary tube, heated at 10° min^{-1} after insertion at 200°) lit.,[4] 214—215° and 224—225°); $\nu_{max.}$ (hydrochloride) 1 675 cm^{-1} (CO).

Cyclisation of 2-[N-(3,4-*dimethoxyphenethyl*)-4-*methoxybenzylamino*]*acetonitrile* (6). *'Hot' cyclisation.* The dried chloroform extract, on removal of the solvent and recrystallisation from petroleum (b.p. 60—80 °C), gave **1,2,3,4-*tetrahydro*-6,7-*dimethoxy*-2-(4-*methoxyphenacyl*)*isoquinoline*** (11) (1.7 g, 33%), m.p. 121°, as pale yellow needles; $\nu_{max.}$ 1 675 cm^{-1} (CO); δ 8.04 (2 H, d, *J* 8 Hz) and 6.88 (2 H, d, *J* 8 Hz, $MeOC_6H_4$), 6.56 (1 H, s, ArH), 6.47 (1 H, s, ArH), 3.88 (2 H, s, $COCH_2N$), 3.79—3.82 (9 H, 3s, 3MeO), 3.70 (2 H, s, $ArCH_2N$), and 2.74 (4 H, s, $ArCH_2 \cdot CH_2 \cdot N$); *m/e* 341 ($M^+$, 6%), 206 (100), 192 (60), 135 (26), and 107 (12) (Found: C, 70.5; H, 6.7; N, 4.1. $C_{20}H_{23}NO_4$ requires C, 70.4; H, 6.7; N, 4.1%).

Cyclisation of 1-(2,3-*dimethoxybenzylamino*)*cyclohexanecarbonitrile* (7). *'Hot' cyclisation.* The dried chloroform extract on removal of solvent and recrystallisation from petroleum (b.p. 80—100 °C) gave 2,3-*dihydro*-5,6-*dimethoxy*-3-*spirocyclohexylisoquinoline*-3-*spirocyclohexan*-4(1H)-*one* (12) (0.25 g, 5%), m.p. 106°; $\nu_{max.}$ 3 250 (NH) and 1 660 cm^{-1} (CO); δ 7.05 (1 H, d, *J* 8 Hz, ArH), 6.80 (1 H, d, *J* 8 Hz, ArH), 3.9 (2 H, s, $ArCH_2N$), 3.80 (6 H, s, 2MeO), 1.95 (1 H, s, exch. NH), and 1.70 (10 H, m, C_5H_{10}); *m/e* 275 (M^+, 30%), 247 (14), 232 (3), 178 (48), 151 (100), and 150 (8) (Found: C, 70.0; H, 7.7; N, 5.2. $C_{16}H_{21}NO_3$ requires C, 69.8; H, 7.7; N, 5.1%).

Attempted cyclisation of 1-(3-*methoxybenzylamino*)*cyclohexanecarbonitrile* (4). *'Hot' cyclisation.* The aminonitrile (4) (5 g), after dissolution in concentrated sulphuric acid, dilution, basification, and extraction with chloroform gave only a trace of intractable gum. The extracted aqueous solution was acidified to pH 7 (pH meter) and after two days white crystals (4 g) appeared. These were collected, recrystallised from distilled water and dried; m.p. 305° (micro-hot-stage; uncorrected); $\nu_{max.}$ 1 090 and 1 160 (SO_2 str.), 1 660 (CO amide), and 3 150 and 3 400 cm^{-1} (NH_2 amide); δ 9.10 (2 H, s, $CONH_2$), 7.0—7.80 (5 H, m, ArH_3, NH, and SO_3H), 4.30 (2 H, s, $ArCH_2$), 3.80 (3 H, s, MeO), and 1.4—2.2 (10 H, m, C_5H_{10}) (Found: C, 52.35; H, 6.45; N, 7.9; S, 9.1. $C_{15}H_{22}N_2O_6S$ requires C, 52.5; H, 6.45; N, 8.2; S, 9.35%).

Synthesis of **1,2,3,4-*Tetrahydro*-6,7-*dimethoxy*-2-(4-*methoxyphenacyl*)*isoquinoline*** (11).—2-Bromo-4′-methoxyacetophenone (5.95 g, 0.026 mol) dissolved in absolute ethanol (50 ml) was added dropwise to a refluxing mixture of 1,2,3,4-tetrahydro-6,7-dimethoxyisoquinoline (5 g, 0.026 mol) and anhydrous sodium carbonate (5 g) in absolute ethanol (50 ml). After 8 h the mixture was filtered and cooled giving pale yellow crystals. Recrystallisation from absolute ethanol gave the product (4 g, 45%) as pale yellow needles, m.p. 121° (mixed m.p. 121°).

[7/1304 *Received*, 20*th July*, 1977]

[4] G. Grethe, H. L. Lee, M. Uskokovic, and A. Brossi, *J. Org. Chem.*, 1968, **33**, 491.

Cyclisation of Benzylamino-nitriles. Part 3.[2] Synthesis of 3-Imidazolines

J. Chem. Research (S), 1978, 154
J. Chem. Research (M), 1978, 1954–1974

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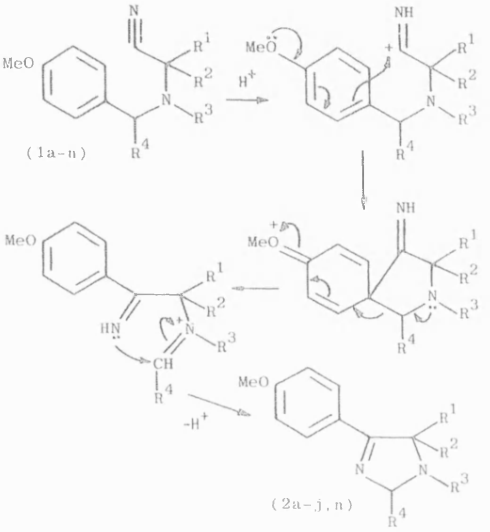
Nucleophilic attack on the imino-iminium ion produced by treatment of (methoxybenzylamino)-acetonitriles with sulphuric acid has been shown to produce tetrahydroisoquinolinones.[2] We now report that, with 4-methoxybenzylaminoacetonitriles (1), the absence of an alternative nucleophile allows only the addition of the imine to the iminium ion, with the formation of a 3-imidazoline ring (2). This is due, we assume, to the low reactivity of the aromatic nucleus in the *ortho*-positions (relative to the imino group) and the high reactivity of the iminium ion. In accord with the work of Kirchner,[3] the 5-monosubstituted imidazoline (2k) was found to be unstable.



	R^1	R^2	R^3	R^4
a	$[CH_2]_5$		H	H
b	$[CH_2]_4$		H	H
c	Et	Et	H	H
d	Et	Me	H	H
e	Me	Me	H	H
f	$[CH_2]_5$		Me	H
g	$[CH_2]_4$		Me	H
h	Me	Me	Me	H
i	$[CH_2]_5$		Bzl	H
j	$[CH_2]_5$		H	Bzl
k	Me	H	H	H
l	H	H	H	H
m	$[CH_2]_5$		DMBzl	H
n	$[CH_2]_5$		ClBzl	H

Bzl = benzyl DMBzl = veratryl
ClBzl = 4-chlorobenzyl

Scheme 1

Techniques used: I.r., ^{1}H n.m.r., mass spec.

References: 5

Tables 1–6: Spectroscopic data, m.p.s, and CHN analyses of 4-methoxybenzylaminoacetonitriles (Tables 1–3) and 3-imidazolines (Tables 4–6)

Scheme 2: General pattern of electron-impact-induced fragmentation of 3-imidazolines

Scheme 3: Detailed fragmentation pattern of the imidazoline (2d)

Scheme 4: Postulated mode of decomposition of the imidazoline (2k)

Scheme 5: Fragmentation pathway of a pyrazine derivative derived from the imidazoline (2k)

Paper: E/016/78

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References cited in this synopsis:

[2] Part 2, D. N. Harcourt, N. Taylor, and R. D. Waigh, *JCS Perkin I*, in the press.
[3] G. Kirchner, *Annalen*, 1959, **625**, 104.

Cyclisation of Benzylamino-nitriles. Part 4.[1] Rearrangement with Cyclisation to a Benzyl or Phenethyl Substituent

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Roger D. Waigh,* Department of Pharmacy, University of Manchester, Manchester M13 9PL

Methoxybenzylamino-acetonitriles with benzyl or phenethyl substituents attached to the nitrile α-carbon undergo spiro-cyclisation in concentrated sulphuric acid followed by rearrangement with cyclisation to the benzene ring of the substituent. The product is a tetrahydroisoquinoline or a 2-benzazepine depending on the substituent.

We have shown[1] that methoxybenzylamino-acetonitriles produce a range of products when treated with concentrated sulphuric acid, all consistent with an initial spiro-cyclisation. One observation which led us to propose this mechanism was the production of a 3-benzoyltetrahydroisoquinoline (2a) rather than the expected 3-benzylisoquinolin-4(3*H*)-one (3a) from the nitrile (1a) (Scheme 1). Here we describe further examples of this kind of rearrangement, and details of the original example.[2]

If the spiro-intermediate (4) is formed as described, only the *para*-methoxy substituent should be required to produce a rearrangement product. This was confirmed by cyclisation of four nitriles (1b—e) which all gave the expected products (2b—e) in moderate to good yield and a high state of purity. As in the case of the original dimethoxybenzoyl rearrangement product (2a), spectral data were unambiguous. The presence of the methoxybenzoyl moiety is clear from the ^{1}H n.m.r. spectra, with typical AA'XX' coupling in the case of the monomethoxy-compounds, and fragmentation with loss of this moiety provides the base peak in the mass spectra.

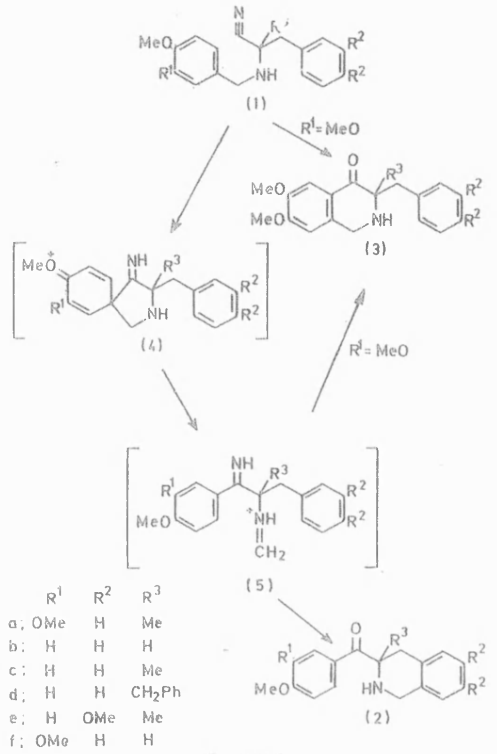
Where the amino-nitrile is derived from veratrylamine [*i.e.* (1; R^1 = OMe)] there is a possibility of straightforward *ortho*-cyclisation to give an isoquinolone (3). It is also possible to arrive at the same product after spiro-cyclisation, if the cyclisation to the imino- and methoxy-substituted ring is energetically comparable with cyclisation to the competing alternative. For an isoquinolone derived from an unlabelled C_α-achiral or C_α-racemic veratrylamine it is not possible to distinguish between the two mechanisms. The amino-nitrile (1f) produced equal amounts of the two products (2f) and (3f), the latter probably[1] by a combination of both mechanisms. Alteration of reaction conditions (temperature, concentration of acid) did not greatly affect the proportions of the two products. Separation was achieved by fractional crystallisation of the hydrobromide salts, other approaches having failed.

When the aminonitrile α-substituent was increased in

[1] Part 2, D. N. Harcourt, N. Taylor, and R. D. Waigh, *J.C.S. Perkin I*, 1978, 722; Part 3, *J. Chem. Research*, 1978, (*S*) 154; (*M*) 1954.

[2] D. N. Harcourt, N. Taylor, and R. D. Waigh, *J.C.S. Chem. Comm.*, 1972, 644.

length to phenethyl, as in structures (6a and b), the rearrangement process and concomitant cyclisation gave a 2-benzazepine [(7a and b), respectively] (Scheme 2). It



	R^1	R^2	R^3
a,	OMe	H	Me
b;	H	H	H
c;	H	H	Me
d;	H	H	CH_2Ph
e;	H	OMe	Me
f;	OMe	H	H

SCHEME 1

is interesting that a perfectly straightforward alternative carbocyclic reaction is possible, particularly with the amino-nitrile (6b), which has a second activated ring, to give a 1-tetralone (8) (Scheme 2). Similar cyclisations in concentrated sulphuric acid are known,[3] but in the present example only the 2-benzazepine was isolated, indicating the surprisingly low energy associated with the nitrogen-containing five-membered spirocyclic system. It is significant in this respect that where the secondary cyclisation was not aided by an activating group, as in (6a), the major product was a sulphonic acid (9), produced by rearrangement but without secondary cyclisation.

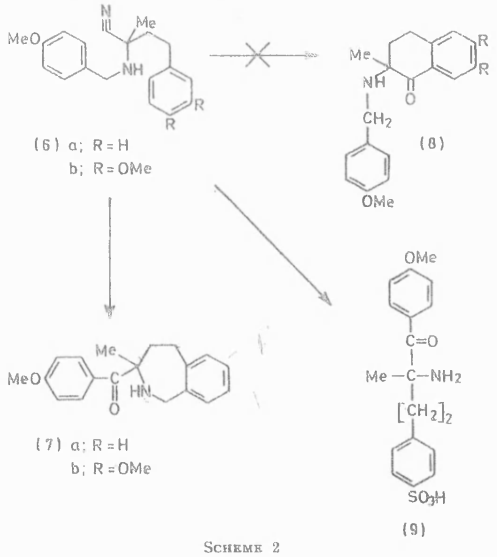
As with the other products reported here, the structures of the benzazepines (7a and b) are confirmed by n.m.r. and mass spectral data, elemental analysis, and i.r. data, while the sulphonic acid is assigned structure (9) from elemental analysis and i.r. and n.m.r. spectral data only, since it was not sufficiently volatile for mass spectrometry. In each case the most significant structural feature is the *p*-methoxybenzoyl moiety, which is clearly defined in both the ^1H n.m.r. and mass spectra (where feasible). The sulphonic acid (9) shows a second AA'XX' aromatic system in the ^1H n.m.r. spectrum, for the sulphonated ring.

Together, these results suggest that spiro-cyclisation is often more favourable than the alternative *ortho*-cyclisation, even where the latter leads to a six-membered ring. This tends to support the assertion[4] that ' reactions which involve spirocyclic intermediates are general. Such reactions may be expected whenever a compound containing a suitably substituted aromatic ring is subjected to reaction conditions which allow for aromatic participation.'

EXPERIMENTAL

M.p.s were taken on a Kofler hot-stage apparatus. I.r. spectra were recorded with a Unicam SP 200 or Perkin-Elmer 137 spectrophotometer for potassium bromide discs or liquid films. N.m.r. spectra were determined for solutions in deuteriochloroform using Perkin-Elmer R12 or Varian HA 100 instruments. Mass spectra were obtained using A.E.I. MS12 or MS902 spectrometers. Mass spectral ion formulae were obtained by computer matching of accurate masses.

Cyclisations were performed as previously.[5] Hydrochlorides were prepared by addition of ethereal hydrogen chloride to an organic solution of the base.



SCHEME 2

Preparation of Amino-nitriles.—Compounds (1a—f) and (6a and b) were prepared as previously described.[5] All

[3] C. K. Bradsher, E. D. Little, and D. J. Beavers, *J. Amer. Chem. Soc.*, 1956, **78**, 2163.

[4] M. S. Newman, *Accounts Chem. Res.*, 1972, **5**, 354.

[5] D. N. Harcourt and R. D. Waigh, *J. Chem. Soc.* (*C*), 1971, 987.

free bases showed weak $-C\equiv N$ i.r. absorption at *ca.* 2 240 cm^{-1}; secondary bases had >NH absorption at *ca.* 3 300 cm^{-1}. ^1H N.m.r. data are given in Table 1, analyses in Table 2. The preparation of the amino-nitrile (6b) was

TABLE 1

^1H N.m.r. data for benzylamino-nitriles (δ values, *J* in Hz)

(1a)	1.45 (3 H), 1.59 (1 H), 3.00 (2 H), 3.85 (8 H), 6.84 (3 H, m), 7.30 (5 H)
(1b)	1.61 (1 H),[a] 2.94 (2 H, d, *J* 6 Hz), 3.53—4.05 (3 H, m), 3.67 (3 H), 6.78 (2 H, d, *J* 8 Hz), 7.17 (d, 2 H, *J* 8 Hz), 7.22 (5 H)
(1c)	1.34 (3 H), 1.54 (1 H),[a] 2.89 (2 H), 3.64 (3 H), 3.76 (2 H), 6.70—6.79 (2 H, d, *J* 9 Hz), 7.08—7.30 (7 H, m)
(1d)	2.64 (1 H),[a] 2.93 (4 H), 3.66 (3 H), 3.77 (2 H), 6.76 (2 H, d, *J* 8 Hz), 7.12 (2 H, d, *J* 8 Hz), 7.24 (10 H)
(1e)	1.45 (3 H), 2.25 (1 H),[a] 2.90 (2 H), 3.80 (2 H), 3.84 (3 H), 3.87 (6 H), 6.75—7.35 (7 H, m)
(1f) †	3.4—3.7 (2 H, m), 3.86 (6 H), 4.05—4.70 (3 H, m) 6.9—7.9 (8 H, m)
(6a)	1.47 (3 H), 1.50—1.65br (1 H),[a] 1.70—2.15 (2 H, m), 2.62—2.95 (2 H, m), 3.72 (3 H,) 3.80 (2 H), 6.75—7.30 (9 H, m)
(6b)	1.42 (1 H),[a] 1.48 (3 H), 2.0 (2 H, m), 2.7 (2 H, m), 3.6—3.84 (11 H, partly resolved), 6.6—7.30 (7 H, m)

[a] Exchangeable. † Hydrochloride, in C_5D_5N.

TABLE 2

Benzylamino-nitriles

	Yield	M.p.	Found (%)			Required (%)		
	(%)	(*T*/°C)	C	H	N	C	H	N
(1a)	80	82	73.6	6.8	4.3	73.4	6.7	4.5
(1b)	56	166 [a]	67.2	6.1	9.2	67.4	6.3	9.3
(1c)	80	80	76.9	7.2	9.7	77.1	7.1	10.0
(1d)	65	153 [b]						
(1e)	75	*c*, *d*						
(1f)	33	175 [a]	64.8	6.2	8.7	65.0	6.3	8.4
(6a)	65	220 [a]	69.1	6.9	8.3	69.0	7.0	8.5
(6b)	65	*c*	71.4	7.5	8.1	71.2	7.3	7.9

[a] Hydrochloride, from ethanol–ether (m.p. with decomp.). [b] Crude hydrochloride, decomposed on warming in ethanol. [c] Semi-solid. [d] Thermolabile; hydrochloride unstable.

carried out using a boiling water-bath, instead of at room temperature.

Isoquinolines.—*3-(3,4-Dimethoxybenzoyl)-3-methyl-1,2,3,4-tetrahydroisoquinoline* (2a) crystallised from light petroleum (b.p. 60—80°); yield 33%, m.p. 138°, $\nu_{max.}$ 1 660 and 3 260 cm^{-1}, δ 1.55 (3 H), 1.95 (1 H, exchangeable), 2.65 and 3.55 (2 H, ABq, *J* 20 Hz), 3.9 (8 H), 6.85 (1 H, d, *J* 8 Hz), 6.8—7.25 (4 H, m), 7.8 (d, *J* 2 Hz), and 8.25 (dd, *J* 8, 2 Hz), *m/e* 311 (2%, $C_{19}H_{21}NO_3$, M^+), 165 (15, $C_9H_9O_3$), 146 (100, $(C_{10}H_{12}N)$, and 144 (20) (Found: C, 73.6; H, 6.8; N, 4.3. $C_{19}H_{21}NO_3$ requires C, 73.6; H, 6.8; N, 4.5%).

3-(4-Methoxybenzoyl)-1,2,3,4-tetrahydroisoquinoline (2b) was obtained as a pale yellow solid after extraction with ether and recrystallisation from light petroleum (b.p. 80—100°); m.p. 110°, yield 42%, $\nu_{max.}$ 1 675 and 3 300 cm^{-1}, δ 2.23 (1 H), 2.6—3.1 (2 H, m), 3.83 (3 H), 4.12 (2 H), 4.53 (1 H, dd, *J* 6, 10 Hz), 6.90 (2 H, d, *J* 9 Hz), 6.90—7.25 (4 H, m), and 7.95 (2 H, d, *J* 9 Hz), *m/e* 267 (2.2%, $C_{17}H_{17}NO_2$, M^+), 135 (21.2, $C_9H_7O_2$), 132 (100, $C_9H_{10}N$), and 130 (21.3, C_9H_8N) (Found: C, 76.4; H, 6.5; N, 5.2. $C_{17}H_{17}NO_2$ requires C, 76.4; H, 6.4; N, 5.2%).

3-(4-Methoxybenzoyl)-3-methyl-1,2,3,4-tetrahydroisoquinoline (2c) was obtained as white needles from light petroleum (b.p. 60—80°) in 73% yield; m.p. 125°, $\nu_{max.}$ 1 660 and 3 400 cm^{-1}, δ 1.55 (3 H), 1.90 (1 H, exchangeable), 2.65 (1 H, d, *J* 16 Hz), 3.50 (1 H, d, *J* 16 Hz), 3.82 (3 H), 3.91 and 3.94 (2 H, outer lines of AB quartet not discernible), 6.85 (2 H, d, *J* 10 Hz), 6.90—7.20 (4 H, m), and 8.33 (2 H, d, *J* 10 Hz) (Found: C, 76.8; H, 6.7; N, 4.8. $C_{19}H_{19}NO_3$ requires C, 76.9; H, 6.8; N, 5.0%).

3-Benzyl-3-(4-methoxybenzoyl)-1,2,3,4-tetrahydroisoquinoline (2d) was obtained by extraction with chloroform, followed by crystallisation from aqueous ethanol, in 38% yield; m.p. 130°, $\nu_{max.}$ 1 665 and 3 300 cm^{-1}, δ 1.86 (1 H), 2.66 (1 H, d, *J* 16 Hz), 2.98—3.36 (2 H, ABq, *J* 13 Hz), 3.38 (1 H, d, *J* 16 Hz), 3.77 (3 H), 3.85 (2 H), 6.81 (2 H, d, *J* 9 Hz), 6.7—7.3 (9 H, m), and 8.37 (2 H, d, *J* 9 Hz), *m/e* 357 (0.8%, $C_{24}H_{23}NO_3$, M^+), 266 (39.8, $C_{17}H_{16}NO_3$), 222 (100, $C_{16}H_{16}N$), 135 (41.6, $C_9H_7O_2$), 131 (14.3, C_9H_9N), 130 (44.4, C_9H_8N), 91 (21.0, C_7H_7), and 77 (13.4, C_6H_5) (Found: C, 79.8; H, 6.6; N, 4.1. $C_{24}H_{23}NO_3$ requires C, 80.2; H, 6.7; N, 4.1%).

6,7-Dimethoxy-3-(4-methoxybenzoyl)-3-methyl-1,2,3,4-tetrahydroisoquinoline (2e) was obtained as white needles in 30% yield; m.p. 116° [from light petroleum (b.p. 60—80°], $\nu_{max.}$ 3 400 cm^{-1}, δ 1.50 (3 H), 1.74 (1 H), 2.55 (1 H, d, *J* 16 Hz), 3.39 (1 H, d, *J* 16 Hz), 3.74 (2 H), 3.79 (9 H), 6.40 (1 H), 6.56 (1 H), 6.84 (2 H, d, *J* 8 Hz), and 8.37 (2 H, d, *J* 8 Hz), *m/e* 341 (<1%, M^+), 206 (100), and 77 (20) (Found: C, 70.5; H, 6.8; N, 4.3. $C_{20}H_{23}NO_4$ requires C, 70.4; H, 6.7; N, 4.1%).

3-(3,4-Dimethoxybenzoyl)-1,2,3,4-tetrahydroisoquinoline (2f) *and 3-Benzyl-6,7-dimethoxy-1,2-dihydroisoquinolin-4(3H)-one* (3f) were obtained as a 1 : 1 mixture (n.m.r.) (0.74 g, m.p. 84°) from cyclisation of the amino-nitrile hydrochloride (2g). Conversion into hydrobromide salts with gaseous hydrogen bromide in chloroform and precipitation with ether, followed by crystallisation from ethanol, gave crystals, m.p. 240°, which dissolved in water. Basification and extraction produced a gum which after trituration with ether and crystallisation from light petroleum (b.p. 80—100°) gave the *isoquinolinone* (3f), m.p. 120°, $\nu_{max.}$ 1 670 and 3 270 cm^{-1}, δ 2.04 (1 H), 2.65—3.85 (3 H, m), 3.94 (6 H), 4.06 (2 H), 6.65 (1 H), 7.36 (5 H), and 7.62 (1 H) (Found: M^+, 297.138 9. $C_{19}H_{19}NO_3$ requires *M*, 297.136 5), *m/e* 297 (8.3%) and 206 (100, $C_{11}H_{12}NO_2$).

Addition of ether to the mother liquor gave, over several days, a second crop of crystals, m.p. 228°, which after treatment as above and recrystallisation from light petroleum (b.p. 60—80°) gave the *tetrahydroisoquinoline* (2f), m.p. 103°, $\nu_{max.}$ 1 675 and 3 300 cm^{-1}, δ 2.83 (1 H), 2.80—3.10 (2 H, m), 3.93 (6 H), 4.19 (2 H), 4.62 (1 H, dd, *J* 6, 10 Hz), 6.91 (1 H, d, *J* 9 Hz), 7.12 (4 H, m), and 7.54—7.78 (2 H, m) (Found: M^+, 297.135 6. $C_{19}H_{19}NO_3$ requires *M*, 297.136 5), *m/e* 297 (6.2%), 165 (8.4, $C_9H_9O_2$), 132 (100, $C_9H_{10}N$), 131 (11.5, C_9H_9N), and 130 (29.4, C_9H_8N). A peak at *m/e* 206 (13.3%, $C_{11}H_{12}NO_2$) was presumed to arise from a small amount of isoquinolinone (3).

3-(4-Methoxybenzoyl)-3-methyl-2,3,4,5-tetrahydro-1H-2-benzazepine (7a).—This was obtained from the amino-nitrile (6a) in 5% yield as a *semi-solid* which would not crystallise, $\nu_{max.}$ 1 665 and 3 400 cm^{-1}, δ 1.10 (3 H), 1.20—1.30 (1 H, m), 1.50 (1 H, exchangeable), 2.3—3.3 (3 H, m), 3.48 (2 H), 3.60 (3 H), 6.69 (2 H, d, *J* 9 Hz), 6.70—7.0 (4 H, m), and 8.30 (2 H, d, *J* 9 Hz), *m/e* 295 (<1%, M^+), 160 (100), and 135 (20) (Found: C, 77.3; H, 7.2; N, 4.5. $C_{19}H_{21}NO_2$ requires C, 77.3; H, 7.1; N, 4.75%).

4-[3-Amino-3-(4-methoxybenzoyl)butyl]benzenesulphonic Acid (9).—This was obtained from the amino-nitrile (6a) by reacidification of the cyclisation medium to pH 6.5 with

hydrochloric acid after basification and extraction. After several days white crystals were filtered off and recrystallised from water to yield the *sulphonic acid* (9), 30%, m.p. 220° (decomp.), $\nu_{max.}$ 690, 850, 1 010, 1 170, 1 665, and 3 400 cm^{-1}, δ(D_2O–CF_3CO_2H) 2.0 (3 H), 2.30—3.00 (4 H, m), 3.94 (3 H), 7.0—7.2 (4 H, 2d overlapping, *J* 8.5 Hz), 7.75 (2 H, d, *J* 8.5 Hz), and 8.05 (2 H, d, *J* 8.5 Hz) (Found: C, 57.6; H, 5.85; N, 3.5; S, 8.8. $C_{15}H_{21}NO_5S,0.5H_2O$ requires C, 58.05; H, 6.0; N, 3.8; S, 8.6%).

7,8-*Dimethoxy*-3-(4-*methoxybenzoyl*)-3-*methyl*-2,3,4,5-*tetrahydro*-1H-2-*benzazepine* (7b).—This was obtained from the amino-nitrile (6b) in 50% yield as fine white needles [from light petroleum (b.p. 60—80°)], m.p. 116°, $\nu_{max.}$ 1 665 and 3 400 cm^{-1}, δ 1.42 (3 H), 1.50—1.61 (1 H, m and 1 H, exchangeable), 2.60—3.0 (3 H, m), 3.63 (2 H), 3.82 (3 H), 3.86 (6 H), 6.56 (1 H), 6.70 (1 H), 6.92 (2 H, d, *J* 9 Hz), and 8.28 (2 H, d, *J* 9 Hz) [irradiation of the multiplet (δ 2.60—3.0) reduced the signal at δ 1.50—1.61 to a singlet], *m/e* 355 (1% M^+, $C_{21}H_{25}NO_4$), 312 (6, $C_{19}H_{22}NO_3$), 220 (100, $C_{13}H_{18}NO_2$), 203 (16, $C_{13}H_{15}O_2$), and 135 (24) (Found: C, 71.4; H, 7.1; N, 3.7%. $C_{21}H_{25}NO_4$ requires C, 71.0; H, 7.4; N, 3.9%).

We thank Mr. P. L. Hillis for experimental assistance.

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808 Chemistry and Industry, 21 October 1978

Oxidative *C*-debenzylation of *N*-benzylpapaverinium bromide under mild alkaline conditions

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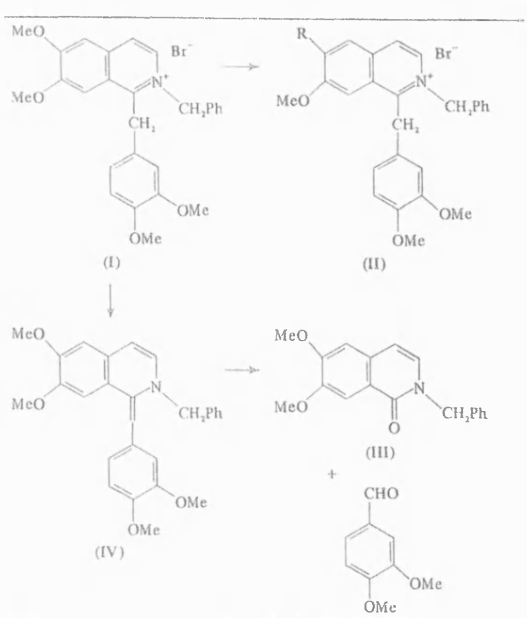
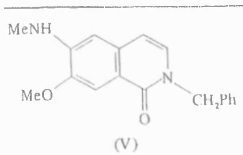
Reaction of amines with 6- or 8-alkoxyisoquinolinium salts has been reported to give high yields of 6- or 8-alkylamino-isoquinolinium salts as appropriate.[1] Whereas the authors have been able to improve these reactions using pyrrolidine or benzylamine with *N*-benzylpapaverinium bromide (I) to obtain amines (II), reaction with diethylamine did not proceed readily under the usual conditions. Prolonged heating was required for significant changes to be detectable by t.l.c., and work-up then gave the isocarbostyril (III, 35 per cent after recrystallisation from ethanol, mp 163–164°C, lit.[2] 162–163°C) as the only crystallisable product. Since this seemed most likely to result from air oxidation of the benzylidene isoquinoline (IV) occurring in the alkaline solution, rather than a specific effect of diethylamine, the reaction was repeated with potassium hydroxide as base. Under reflux in *t*-butanol in a system flushed with nitrogen no reaction occurred, but occasional bubbling with oxygen gave a 95 per cent yield of isocarbostyril (III). It was also possible to identify veratraldehyde as the other oxidation product by isolation as the dinitrophenylhydrazone and comparison with an authentic sample.

During the course of this work the air oxidation of similar





(V)

papaverinium salts in alkaline solution was reported[3] using either copper(II) chloride or ultraviolet light with a free-radical initiator. None of the extra catalysts appears to be necessary.

Presumably the lack of reactivity of diethylamine in the alkoxy-substitution reaction is attributable to a slight increase in effective bulk compared with pyrrolidine, an effect further exemplified by the difference in reactivity between methylamine and dimethylamine. Both undergo reaction at room temperature over a period of several days, the former to give the amine (II, R = MeNH, mp 200–202°C, 76 per cent yield), the latter to give dimethylamine hydrobromide as well as the isocarbostyril (III) and veratraldehyde, with no indication of any other products on t.l.c. All three products were identified by ^{1}H n.m.r. spectroscopy.

Amino-isocarbostyril has not been detected in reactions producing amino-isoquinolinium salts (II), which may be attributable to resonance stabilisation of the positive charge between the two nitrogens. However, under more forcing conditions (potassium hydroxide and oxygen), the quaternary salt (II, R = MeNH) was converted cleanly into the amino-isocarbostyrils (V), mp 153–156°C, $\nu_{max.}$ 1640 (C=O); δ 2·92 (3H, *d* (broad), J = 4Hz, NHCH_3); 3·92 (3H, OCH_3); 4·8 (1H, broad, N*H*); 5·19 (2H, NCH_2 Ph); 6·35 (1H, *d*, J = 7Hz, C4*H*); 6·43 (1H C5*H*); 7·28 (5H, Ar); 7·70 (1H, C8*H*). (Found: M$^+$; 294·1366. $C_{18}H_{18}N_2O_2$ requires M; 294·1368).

Monitoring of the reactions by t.l.c. was carried out using silica with butanol (70)/acetic acid (10)/water (25 parts by volume) as eluting solvent. Isocarbostyrils and veratraldehyde were separated on silica with ether as eluting solvent. Spots were visualised with aqueous potassium permanganate or iodochlorplatinate reagent. Melting points are corrected.

The SRC is thanked for an award to D.B.

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References

[1] Naruto, S., Mizuta, H. & Nishimura, H., *Tetrahedron Lett.*, 1976, 1597

[2] Battersby, A. R., Davidson, G. C. & Turner, J. C., *J. chem. Soc.*, 1961, 3899

[3] Ruchirawat, S., Borvornvinyanant, U., Hantawong K. & Thebtaranonth, Y., *Heterocycles*, 1977, 6, 1119

332 J. CHEM. RESEARCH (S), 1979

Bischler–Napieralski Cyclisation of *N*-[2-(2-Naphthyl)ethyl] amides

J. Chem. Research (S), 1979, 332
J. Chem. Research (M), 1979, 3864–3877

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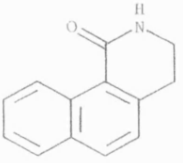
Electronic factors favour angular cyclisation of naphthylethylamine derivatives (1), but steric interactions between R^1 and R^3 in the product (3) have an opposing effect. Thus the dimethoxy analogue (1a) gave[5] the angular dihydrobenz[*h*]isoquinoline (3a), but the isomer (1b) gave[6] the linear dihydrobenz[*g*]isoquinoline (2b). The first reports of such cyclisations[1] assigned, for example, the linear structure to the product from amide (1g) but acknowledged the inadequacy of the data available at the time in distinguishing linear from angular isomers.

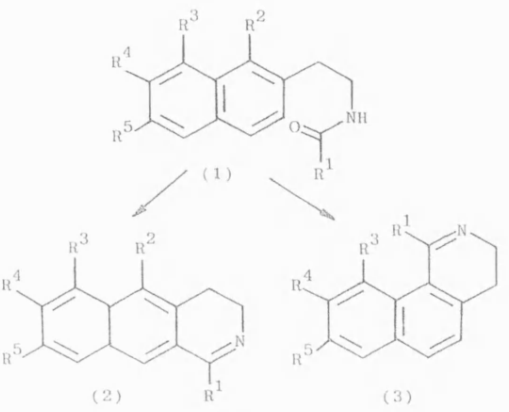
We have cyclised a series of amides under typical Bischler–Napieralski conditions, and have obtained good yields of angular isomers (3) rather than the linear products (2), the distinction being made by means of the aromatic coupling constants in the ^{1}H n.m.r. spectra, clarified by the use of Eu(fod)$_3$ as shift reagent.

Angular cyclisation occurred despite acute steric interactions in the diphenylacetamide (1e), and with expulsion of the blocking group (1h, i, j) where attempts were made to force linear cyclisation.

Attempts were made to isomerise the angular isoquinolinone (4) [from amide (1f)] by analogy with other work,[9] but without success.



(4)



(1) (2) (3)

	R^1	R^2	R^3	R^4	R^5
a	H	H	H	OMe	OMe
b	H	H	OMe	OMe	H
c	H	H	H	H	H
d	CH_2Ph	H	H	H	H
e	$CHPh_2$	H	H	H	H
f	OEt	H	H	H	H
g	Ph	H	H	H	H
h	H	Br	H	H	H
i	CH_2Ph	Br	H	H	H
j	H	SEt	H	H	H

Techniques used: I.r., ^{1}H n.m.r., mass spec.

References: 17

Paper: E/110/79

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References cited in this synopsis:

[1] K. Kindler, W. Peschke, and G. Plüddemann, *Arch. Pharm.*, 1939, 277, 25.
[5] K. Y. Zee-Cheng, W. H. Nyberg, and C. C. Cheng, *J. Heterocycl. Chem.*, 1972, 9, 805.
[6] C. S. Menon, K. Y. Zee-Cheng, and C. C. Cheng, *J. Heterocycl. Chem.*, 1977, 14, 905.
[9] I. Agranat and Y.-S. Shih, *Synthesis*, 1974, 865.

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Chemistry and Industry, 5 April 1980

Amine substitution in quaternary isoquinolinium salts

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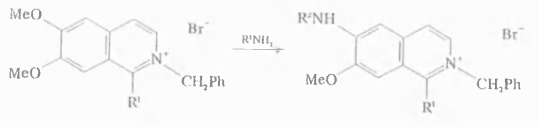
Quaternary isoquinolinium salts have been reported to undergo substitution in which a 6- or 8-methoxy group is replaced by an amino function on treatment with the appropriate amine.[1,2] Now, it has been found that, whereas the quaternary salt (I*a*) gives the expected product (II*a*) on treatment with benzylamine and salt (I*b*) gives the expected product (II*b*) on treatment with methylamine (see Scheme 1), salt (I*a*) undergoes an unexpected double substitution with methylamine to give (III), with the alternative monosubstitution products (IV) and (V) as intermediates (see Scheme 2).

2-Benzyl-6,7-dimethoxy-1-isopropylisoquinolinium bromide (I*b*), mp 157—158°C, was boiled with a 20-fold excess of benzylamine in ethanol for 20h to give the 6-benzylaminoisoquinolinium salt (II*a*) in 94 per cent yield, mp 179—180°C, from ethanol-ethyl acetate-ether. 2-Benzyl-6,7-dimethoxyisoquinolinium bromide (I*b*) monohydrate, mp 115—117°C, was treated with a large excess of methylamine in ethanol at room temperature for 8 days to give the 6-methylaminoisoquinolinum salt (II*b*), in 90 per cent crude yield, which recrystallised from ethanol-ethyl acetate-ether as the monohydrate, mp 114—116°C. Heating in a pressure vessel at 100°C for 7 days gave a similar result.

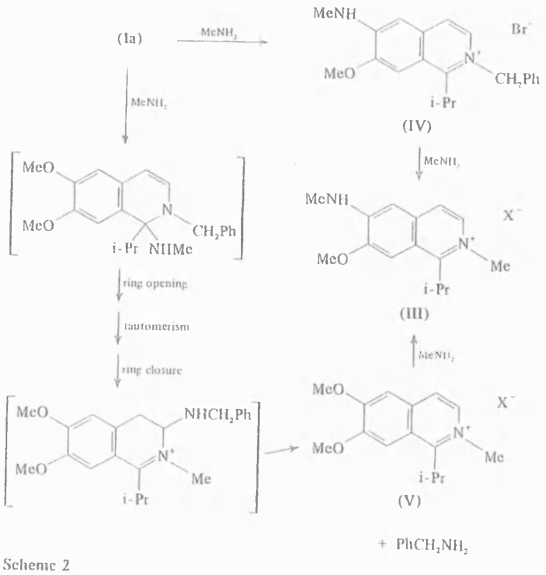
When the 1-isopropyl salt (I*a*) was heated at 100°C with methylamine for 3—11 days, or left at room temperature for 28 days, the major product (61—88 per cent yield) was 1-isopropyl-7-methoxy-2-methyl-6-methylaminoisoquinolinium bromide (III), together with benzylamine (identified by gas chromatography and by conversion into benzyl benzamide) and small quantities of benzyl alcohol and 6,7-dimethoxy-1-isopropylisoquinoline, presumably arising from hydrolysis. The structure of the major product was confirmed by reaction of methylamine with 6,7-dimethoxy-1-



(I) (a), R^1 = i-Pr
(b), R^1 = H

(II) (a), R^1 = i-Pr, R^2 = CH_2Ph
(b), R^1 = H, R^2 = Me

Scheme 1



Scheme 2

isopropyl-2-methylisoquinolinium iodide (V, X = I) which gave the expected 6-methylamino derivative (III), as the iodide. This iodide and the bromide (III, X=Br) from the benzyl quaternary (*Ia*) gave the same tetrahydroisoquinoline (dihydrochloride mp 178—181°C), on reduction with sodium borohydride. Reaction of compound (*Ia*) with methyl amine at room temperature for 64h allowed the isolation of 2-benzyl-1-isopropyl-7-methoxy-6-methylaminoisoquinolinium bromide (IV), mp 211—213°C, in 23 per cent yield, and the identification of 6,7-dimethoxy-1-isopropyl-2-methylisoquinolinium bromide (V), mixed with starting material, by ^{1}H n.m.r. spectral measurements.

The only precedents that can be found are the reactions of *N*-2,4-dinitrophenyl isoquinolinium or pyridinium salts with aniline, which are reported to give *N*-phenyl products.[3]

It is proposed that the first step is attack at C(1) by the amine (Scheme 2). In the present example this would give a highly crowded intermediate; steric strain would be reduced by ring opening and reclosure to give a 3-benzylamino intermediate which would eliminate benzylamine to give the observed product. Relief from steric strain would be much less of a factor in the absence of the 1-isopropyl substituent. Reaction of *N*-benzylisoquinolinium bromide with methylamine produced a dark red viscous gum which was not readily amenable to the isolation of products. However, the presence of benzylamine and *N*-methylisoquinolinium bromide could be demonstrated from ^{1}H n.m.r. spectra and by t.l.c., although the major products were not identified.

All isolated compounds had infrared, ^{1}H n.m.r. and C,H, N analyses consistent with structure; melting points are corrected.

The S.R.C. is thanked for an award to D.B.

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References

1 Naruto, S., Mizuta, H. & Nishimura, H., *Tetrahedron Lett.*, 1976, 1597

2 Beaumont, D. & Waigh, R. D., *Chem. Ind. (London)*, 1978, 808

3 Zincke, T. & Weisspfennig, G., *Liebigs Ann.*, 1913, **396**, 103; 1913, **397**, 255; Zincke, T. & Krollpfeiffer, F., *ibid*, 1915, **408**, 314

Rearrangement of Benzylaminonitriles to give Cyclohepta[*c*]pyrrol-6(2*H*)-ones

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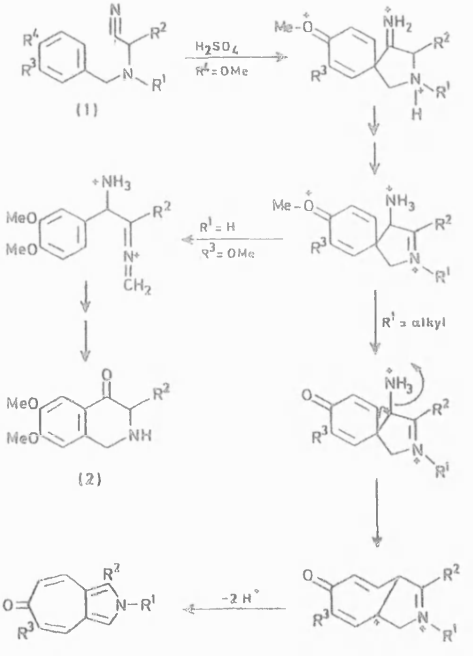
Summary *N*-4-Methoxybenzyl- and *N*-3,4-dimethoxybenzyl-aminoacetonitriles with both 2-aryl and *N*-alkyl substituents undergo *O*-demethylation and rearrangement with elimination of ammonia in concentrated sulphuric acid to give 1-aryl-*N*-alkylcyclohepta[*c*]pyrrol-6(2*H*)-ones.

PREVIOUS work[1] has shown that the benzylaminonitriles (**1a,b**) cyclise in concentrated sulphuric acid to give good yields of the isoquinolinones (**2**), and there is good evidence that a major route of cyclisation in many similar examples[2] is through a spiro intermediate (Scheme). It has now been shown that the *N*-alkyl analogues (**1c,d,e** and **4**) rearrange, probably through a comparable intermediate, to give instead the cyclohepta[*c*]pyrrol-6(2*H*)-ones (**3c,d,e** and **5**) respectively, in 10—50% yield.‡

The difference in behaviour can be tentatively explained if the spiro intermediate (Scheme) is considered in detail. Under the very strongly acidic conditions protonation would probably occur on both nitrogen atoms, with the possibility of tautomerism to give the most stable conjugated iminium salt. Such a salt could undergo cleavage of the five-membered ring to give the intermediate postulated previously, or a tautomer, when the starting amine is secondary (R^1 = H, Scheme), but when R^1 = alkyl this path is blocked. A longer lifetime of the spiro intermediate might reasonably be expected to lead to *O*-demethylation, apparently followed by a 1,2 shift with elimination of ammonia to give the observed products (Scheme).

All analytical and spectral data (i.r., ^{1}H n.m.r., m.s., C,H,N analysis) support the cyclophepta[*c*]pyrrole structures. The coupling constants for the adjacent protons in the seven-membered ring are 12—13 Hz, in accordance with published data on the parent compound,[3] as are the chemical shifts of these protons and those of the pyrrole ring and the *N*-methyl group. The compounds are bright yellow, changing to deep blue or green in acid.

To test the proposed mechanism, the aminonitrile (**1c**) was prepared with potassium [^{13}C]cyanide and cyclised as usual. The product (**3c**) showed enhancement of a previously low-intensity singlet at 119·1 p.p.m. in the ^{13}C n.m.r. spectrum, with no splitting in the off-resonance decoupled spectrum, in accordance with expectation for a carbon in the ring junction.

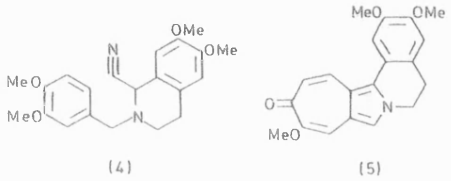


	R^1	R^2	R^3	R^4
a	H	Ph	OMe	OMe
b	H	DMP	OMe	OMe
c	Me	Ph	OMe	OMe
d	Me	DMP	OMe	OMe
e	Me	Ph	H	OMe
f	Me	Ph	OMe	H

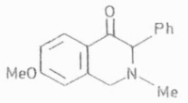
DMP = 3,4-dimethoxyphenyl

SCHEME†

The remaining concern was with the position of the methoxy-group in the seven-membered ring of compounds (**3c**,**d** and **5**). It would be expected on mechanistic grounds that the preferred isomer would be as depicted since only then could the methoxy group stabilise the positive charge developing on the spiro carbon during the 1,2-shift. This supposition is borne out by the large (0·6 p.p.m.) chemical shift difference shown by the proton β to the carbonyl (J 13 Hz) in (**3c**) and (**3d**) compared to (**5**), which would only be expected if this proton could be deshielded by the rigidly held aromatic ring in (**5**).



(4) (5)



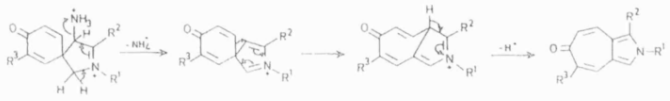
(6)

It has been noted previously that straightforward *ortho*-cyclisation with a single activating methoxy-group is not favoured,[4] and this was borne out by cyclisation of the nitrile (**1f**) which gave 10—12% yields of the isoquinolinone (**6**) and was otherwise largely sulphonated, without cyclisation.

P. Baker and P. L. Hillis are thanked for experimental assistance.

(*Received, 7th August* 1980; *Com.* 873.)

† A referee has suggested that elimination of ammonia could precede ring expansion:



‡ At least 95% of pure recrystallized material except for the product from (**1e**).

[1] D. N. Harcourt and R. D. Waigh, *J. Chem. Soc.* (*C*), 1971, 967.
[2] D. N. Harcourt, N. Taylor, and R. D. Waigh, *J. Chem. Soc., Perkin Trans.* 1, 1978, 1330 and references cited.
[3] J. Duflos, D. Letouzé, G. Queguiner, and P. Pastour, *Tetrahedron Lett.*, 1973, 3453.
[4] D. N. Harcourt, N. Taylor, and R. D. Waigh, *J. Chem. Soc., Perkin Trans.* 1, 1978, 722.

SHORT COMMUNICATION

Differentiation of Isomeric 1,2-Dihydroisoquinolin-4(3*H*)-ones by Proton Magnetic Resonance of their 4-Benzyl Derivatives

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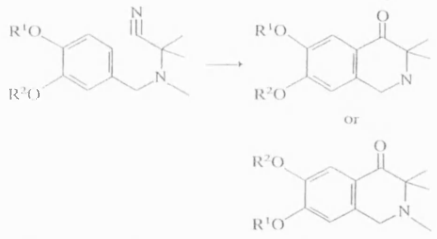
Reaction of a 3,3-dimethyl-1,2-dihydroisoquinolin-4(3*H*)-one with a benzyl Grignard reagent gives a 4-benzyltetrahydroisoquinoline in which the signal of a 6-methoxy group suffers a large upfield shift in the ^{1}H NMR spectrum, whereas a 7-methoxy group is relatively unaffected. This effect can be used to determine whether an RO-substituent is in the 6- or 7-position and, in particular, to distinguish isomeric derivatives with different RO-groups in the 6- and 7-positions. The method will allow the mechanism of cyclization of 3,4-dimethoxybenzylaminoacetonitriles to be more fully elucidated.

It has been shown that benzylaminoacetonitriles undergo cyclization in concentrated sulphuric acid to give 1,2-dihydroisoquinolin-4(3*H*)-ones[1] and that cyclization can proceed both by straightforward *ortho* attack, and by rearrangement[2] (Scheme 1). Where the starting material is a 3,4-dioxygenated benzylamine, the two routes of cyclization will give different 6,7-dioxygenated 1,2-dihydroisoquinolin-4(3*H*)-ones, unless both oxygen substituents are the same. A method for distinguishing the isomers where the oxygen substituents are different will be extremely useful, both to identify reaction products and to investigate the mechanism of cyclization.



Scheme 1. Alternative courses of cyclization of benzylaminoacetonitriles.

As may be seen from the data given here, neither the ^{1}H NMR spectra of a representative ketone, **1**, nor its reduction product, **2**, allow any distinction to be made between 6- and 7-methoxy groups, although there is the expected upfield shift of H-5 caused by loss of the anisotropic deshielding effect of the carbonyl group (see Fig. 1).

^{1}H NMR spectra of 1-benzyl-1,2,3,4-tetrahydroisoquinolines have been studied in considerable detail.[3] The conclusion reached is that alkyl substituents vicinal to the benzyl group in the heterocyclic ring force the benzyl group to adopt a conformation in which the aromatic ring lies under the 7- and 8-positions of the tetrahydroisoquinoline, with an anisotropic shielding effect which results in substantial upfield shifts of the signals of H-8 and the 7-alkoxy group.

It was apparent that the introduction of a benzyl group into the 4-position of a 3-substituted tetrahydroisoquinoline should produce a sterically analogous situation, with an upfield shift of H-5 and the 6-alkoxy group. This was specifically accomplished in the present instance by reaction with a benzyl Grignard reagent to give the 4-hydroxytetrahydroisoquinoline **3**. The induced shifts were even larger than those observed for 1-benzyltetrahydroisoquinolines,[3] and will allow structure determinations where $R^1 \neq R^2$ to be made with total confidence. In order to ensure that the effect is attributable to the anisotropic effect, and is not caused by a 4-alkyl substituent in any other way, the methyl Grignard adduct **4** was also prepared: the shift data are conclusive.

As an example of the application of the method, the partially demethylated isoquinolinone **5**, isolated from cyclization of the appropriate 3,4-dimethoxybenzylaminoacetonitrile, was considered. From NMR data on the ketone itself two isomers were possible, but reaction with benzylmagnesium chloride gave adduct **6**, for which the chemical shift of the O—CH_3 substituent shows conclusively that it is in the 6-position.

EXPERIMENTAL

Spectra were obtained on a Perkin-Elmer R12B spectrometer, using 10% solutions in deuterochloroform with tetramethylsilane as internal standard.

Acknowledgements

I thank M. R. Euerby and C. Green for experimental assistance.

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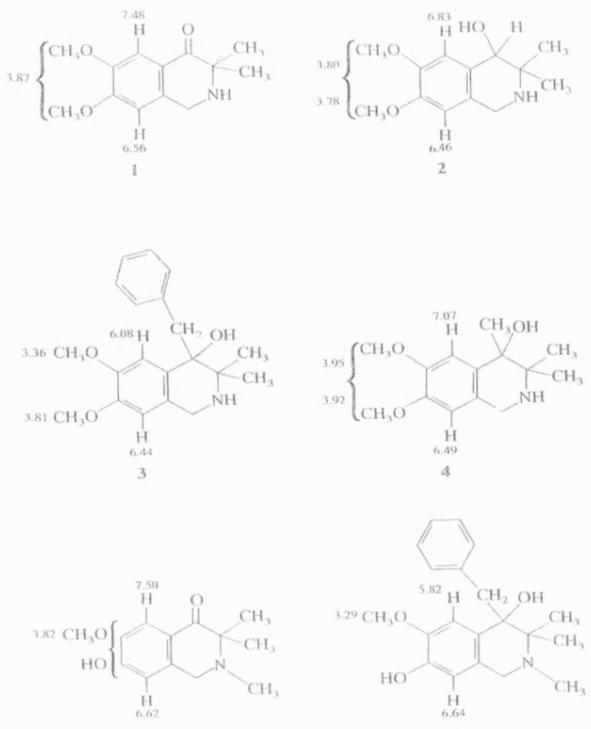

Figure 1. Significant ^1H chemical shifts (δ values in ppm from TMS) of isoquinolinones and isoquinolinols.

REFERENCES

1. D. N. Harcourt and R. D. Waigh, *J. Chem. Soc. C*, 967 (1971).
2. D. N. Harcourt, N. Taylor and R. D. Waigh, *J. Chem. Soc. Perkin Trans.* 1, 722 (1978).
3. D. R. Dalton, M. P. Cava and K. T. Buck, *Tetrahedron Lett.* **31**, 2687 (1965); M. Tomita, T. Shingu, K. Fujitani and H. Furukawa, *Chem. Pharm. Bull.* **13**, 921 (1965); L. Radics, M. Kajtar, J. Kobor and G. Bernath, *Acta. Chim. Acad. Sci. Hung.* **60**, 381 (1969).

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FORTHCOMING MEETING

1-14 September 1980

NATO Advanced Study Institute on High Resolution NMR in Solids, Villasimius, Sardinia, Italy.

Further details from:

T. Axenrod, Department of Chemistry, The City College of CUNY, New York, New York 10031, USA.
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SYNTHETIC COMMUNICATIONS, 11(10), 849-851 (1981)

A CONVENIENT SYNTHESIS OF 3-METHYLTHIOBENZALDEHYDE

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Requiring the title compound as a starting material, we were surprised to find only one published synthesis,[1] involving five steps with an overall yield of 15%. This is in contrast to the 2- and 4-alkylthiobenzaldehydes, which may be synthesised in one step by reaction of sodium alkanethiolates with the readily available halobenzaldehydes[2]. 3-Halobenzaldehydes, although readily available, do not react under these conditions[2].

This lack of reactivity was overcome by protecting the aldehyde function, as the acetal, forming the Grignard reagent, and introducing the methylthio substituent by reaction with dimethyl disulphide, in an adaptation of a method reported by Ando and Emoto[3] for a related structure. Deprotection and distillation gave the pure aldehyde in 67% overall yield.

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3-Methylthiobenzaldehyde diethylacetal.

The Grignard reagent was prepared from 3-bromobenzaldehyde diethylacetal (22g) and magnesium (2.6g) in dry THF (200ml), under a N_2 atmosphere, which was maintained while dimethyl disulphide (9.4ml) in dry THF (50ml) was added dropwise, over 30 minutes. The solution was refluxed for 3½ h., cooled and 20% NH_4Cl solution (475ml) added cautiously, keeping the temperature below 20°. Evolution of methanethiol was noted during this process; a trap is advisable. Extraction with ether gave a light brown oil (22g,93%). δ($CDCl_3$)7.51-7.11(4H,m),5.44(1H,s),3.56(4H,q J 7Hz),2.45(3H,s),1.21 (6H,t,J 7Hz), m/e 226(M^+,23%),181(100),153(44),131(6),125(53),77 (21). Measured: M^+226·1026. $C_{12}H_{18}O_2S$ requires M 226·1027.

3-Methylthiobenzaldehyde.

The above acetal (22g) was refluxed with 2% H_2SO_4 (250ml) for 45 min. Work-up gave the aldehyde as a brown oil (14g,95%) which was distilled under a nitrogen atmosphere, b.p. 82-3°/0.4mm Hg (lit[1] 90°/0.4mm Hg), to give 11.1g (75%) of pure aldehyde, δ($CDCl_3$) 9.89(1H,s),7.75-7.23(4H,m),2.47(3H,s), m/e 152(M^+,100%), 151(51), 123(23), 109(10). Measured: M^+152·0254. C_8H_8OS requires 152·0296.

We thank the S.R.C. for an award (to M.R.E.)

References

1. J.W. Baker, G.F.C. Barrett and W.T. Tweed, J.Chem.Soc., 2831 (1952).

2. W.A. Gregory and A. Kreuchunas, U.S. Patent 2761873 (1956), Chem. Abs. 51, 4430e. H.R. Meyer, Brit. Pat. 1416116 (1975), Chem. Abs. 82, 18631. E.M. Sarett and T.Y. Shen, U.S. Patent 3196162, Chem.Abs. 63, 16309. M.R. Euerby and R.S. Waigh, unpublished.

3. M. Ando and S. Emoto, Bull. Chem. Soc. Japan, 51, 2435 (1978).

240 J. CHEM. RESEARCH (S), 1982

Cyclisation of Benzylaminonitriles. Part 5.† Alkylthio and Arylthio Activating Groups

J. Chem. Research (S), 1982, 240–241
J. Chem. Research (M), 1982, 2417–2460

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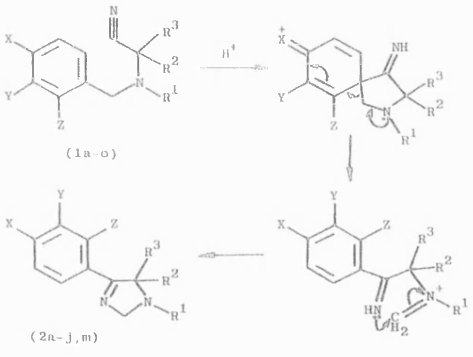
Rearrangements of benzylaminonitriles in sulphuric acid have previously been shown to provide routes to imidazolines[1] and benzazepines[20] and to various isoquinolines.[17,20] The present work shows that alkylthio substituents on the benzene ring are effective activating groups for this kind of reaction, which does not proceed in the absence of an electron-donating group.

To date the only activating group which had been utilised was alkoxy[1,2,17,20] which has the disadvantage when the reaction is applied to the synthesis of specifically required products, *e.g.* in medicinal chemistry, of being very difficult to remove later. We have therefore looked for removable activating groups, which might find application in many other synthetic sequences, using the aminonitrile rearrangement, with which we are familiar, as a test case. So far we have examined only the activating potential of alkylthio and arylthio substituents in this context, without attempting their removal. However, there are many precedents for the removal of sulphur from aromatic rings.[3]

Our results show that *spiro* rearrangement (Scheme 2) to give the imidazoline (2a–h) was effectively activated by a single *para* methylthio or ethylthio substituent; rather longer reaction times were needed than with alkoxy activating groups,[1] but yields were comparable, particularly at room temperature, with concentrated sulphuric acid as cyclising agent. At higher temperatures there was a tendency for *S*-dealkylation to occur, presumably giving the thiol which was isolated as the imidazoline disulphide (3a–d). Surprisingly, the doubly activated aminonitriles (1i and j) required a longer reaction time, perhaps because of steric interactions in the transition state.

Lack of an activating group, as with the aminonitrile (1o), again gave only the amide (5b) by hydrolysis. The amide (12) was also the only product identified when the *para* substituent was benzylthio, as with the aminonitrile (1l).

Phenylthio activating groups, as in (1m and n), gave no extractable product when sulphuric acid was used as cyclising agent. We attribute this to sulphonation, since the phenyl rings are themselves activated by the sulphur atom to which they are attached, and have sterically unprotected *para* positions. To avoid this problem, we investigated the use of polyphosphoric acid as cyclising agent. With the methylthio analogue (1b), we obtained both the imidazoline (2b) and the amide (13), in approximately equal proportions, and a time-profile of the reaction using gas chromatography indicated that

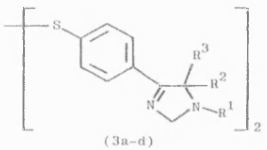


(1a–o)

(2a–j,m)

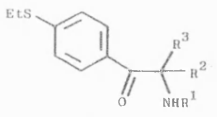
	X	Y	Z	R^1	R^2	R^3
a	MeS	H	H	H	$[CH_2]_5$	
b	MeS	H	H	H	Me	Me
c	MeS	H	H	Me	$[CH_2]_5$	
d	MeS	H	H	Me	Me	Me
e	EtS	H	H	H	$[CH_2]_5$	
f	EtS	H	H	H	Me	Me
g	EtS	H	H	Me	$[CH_2]_5$	
h	EtS	H	H	Me	Me	Me
i	EtS	H	EtS	H	$[CH_2]_5$	
j	EtS	H	EtS	H	Me	Me
k	EtS	H	H	H	Me	$PhCH_2$
l	$PhCH_2S$	H	H	H	Me	Me
m	PhS	H	H	H	$[CH_2]_5$	
n	PhS	PhS	H	H	$[CH_2]_5$	
o	H	H	H	H	$[CH_2]_5$	

Scheme 2



(3a–d)

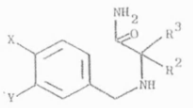
	R^1	R^2	R^3
a	H	$[CH_2]_5$	
b	H	Me	Me
c	Me	$[CH_2]_5$	
d	Me	Me	Me



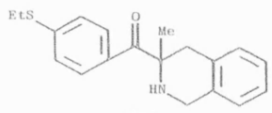
(9f–h)

	R^1	R^2	R^3
f	H	Me	Me
g	Me	$[CH_2]_5$	
h	Me	Me	Me

*To receive any correspondence.
†Part 4 is ref. 20.



	X	Y	R^2	R^3
(5b)	H	H	$(CH_2)_5$	
(12)	$PhCH_2S$	H	Me	Me
(13)	MeS	H	Me	Me
(17)	PhS	H	$(CH_2)_5$	
(19)	PhS	PhS	$(CH_2)_5$	



(11)

longer reaction times would make little difference. With the phenylthio compound (1m) a comparable picture was obtained, but with relatively little imidazoline (2m) formation compared to amide (17). This is consistent with a weaker activating effect from the phenylthio substituent compared to methylthio, and inability of the amide to form a suitable electrophile in polyphosphoric acid for electrophilic attack at the *spiro* position. In accord with these results, the disubstituted nitrile (1n) gave only the amide (19).

It was found previously[20] that a benzyl side-chain could attack the iminium ion after rearrangement to give a 3-benzoyl-1,2,3,4-tetrahydroisoquinoline as the ultimate product. This was also the case with an ethylthio activating group, in the one example studied, where the aminonitrile (1k) gave the tetrahydroisoquinoline (11).

The ready hydrolysis of the imidazolines was exemplified by the conversion of (2f–h) to the aminoketones (9f–h) in dilute hydrochloric acid.

In the full text we have given preparative methods, physical constants, and spectroscopic and analytical data for the benzaldehydes and benzylamines required as intermediates, as several of the aldehydes and all but two of the benzylamines are new.

We thank the S.E.R.C. for an award (to M.R.E.).

Paper: E/090/82 Received: 4th June 1982

Techniques used: I.r., ^1H n.m.r., mass spec., g.c.

References: 28

Table 1: Yields of benzylaminonitriles

Table 2: Yields of 3-imidazolines

Table 3: Relative yields of 3-imidazolines and 3-imidazoline disulphides formed under differing 'hot' cyclisation conditions

Tables 4 and 5: Spectroscopic data for benzaldehydes

Table 6: M.p.s., yields, and reaction conditions for the benzaldehyde oximes

Tables 7–9: B.p.s., m.p.s., and CHN and spectroscopic data for the benzylamines

Tables 10–12: M.p.s. and CHN and spectroscopic data for the benzylaminonitriles

Tables 13–15: Yields, m.p.s., and CHN and spectroscopic data for the 3-imidazolines

Tables 16–19: M.p.s., and CHN and spectroscopic data for the 3-imidazoline disulphides

Tables 20–22: Yields, m.p.s., and CHN and spectroscopic data for the α-aminoketones

Figures 1 and 2: The proportion of reaction products from the cyclisation of aminonitriles (1b) (Figure 1) and (1m) (Figure 2) in polyphosphoric acid at 75 °C *versus* time

Schemes 1 and 2: The postulated mode of formation of 3-imidazolines

Scheme 3: α-Aminoketones synthesised

Scheme 4: The postulated mode of formation of 3-benzoyl-1,2,3,4-tetrahydroisoquinolines

Scheme 5: Polyphosphoric acid cyclisation of the aminonitrile (1b)

Schemes 6 and 7: Postulated mode of formation of products from polyphosphoric acid cyclisations

Schemes 8 and 9: Polyphosphoric acid cyclisations of aminonitriles 1m (Scheme 8) and 1n (Scheme 9)

References cited in this synopsis:

[1]D. N. Harcourt, N. Taylor, and R. D. Waigh, *J. Chem. Research*, 1978, *(S)* 154; *(M)* 1954.
[2]D. N. Harcourt and R. D. Waigh, *J. Chem. Soc. C.*, 1971, 967.
[3]G. R. Pettit and E. E. van Tamelen, *Org. React.*, 1962, 12, 356.
[17]D. N. Harcourt, N. Taylor, and R. D. Waigh, *J. Chem. Soc., Perkin Trans. 1*, 1978, 722.
[20]D. N. Harcourt, N. Taylor, and R. D. Waigh, *J. Chem. Soc., Perkin Trans. 1*, 1978, 1330.

793 J. CHEM. SOC., CHEM. COMMUN., 1982

Rearrangement and Cyclisation of *N*-(2-Hydroxyphenethyl)-2-aminomethylthiophens

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Treatment with acids converted *N*-(2-hydroxy-2-phenethyl)-2-aminomethylthiophens into thienotetrahydropyridines in good yield; with trifluoroacetic acid the major product was the rearranged 7-phenyl-4,5,6,7-tetrahydrothieno[3,2-*c*]pyridine, whereas with polyphosphoric acid the product formed exclusively was the non-rearranged 4-phenyl-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine.

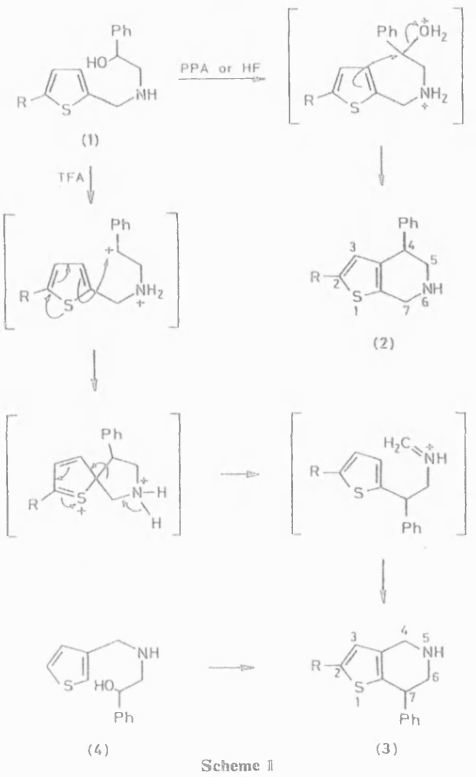
As part of an investigation[1] into *spiro*-rearrangements of the system aryl–C–N–C–C, we have been investigating the claims made in a patent[2] that thiophen derivatives of type (1) cyclise under various acidic conditions (specifically mentioned are phosphoric, polyphosphoric, concentrated sulphuric, and trifluoroacetic acids) to give thienopyridines (2). In our hands, trifluoroacetic acid (TFA) under reflux for 8 h has given clean products with very high yields of cyclised material (Table 1), which ^{1}H n.m.r. spectroscopy has shown to consist of mixtures of the two isomers (2) and (3), the latter predominating (Table 1). We assume that the latter is formed by a spirocyclic rearrangement, as in Scheme 1. Cyclisation of the 3-substituted thiophen (4) gave exclusively the thienopyridine (3; R=H), identical with that obtained by rearrangement from alcohol (1), in accord with the expected position of electrophilic attack on thiophen. The isomers were readily distinguished by the separate resonances of 3-H in (2) and (3), the former showing a relative upfield shift of *ca.* 0.2 p.p.m. owing to shielding by the phenyl ring (Table 1).

We were surprised by a recent report by Maffrand and co-

Table 1.

Starting material	Cyclising agent	Crude yield (%)	Isomer composition (%) (2)	(3)	N.m.r. (3-H, δ)
(1, R = H)	TFA	100	20[a]	80[a]	6.55, 6.76
	PPA	89	100		6.55
	HF	100	100		6.55
(1; R = Me)	TFA	100	11[a]	89[a]	6.24, 6.41
	PPA	100	100		6.24
(4)	TFA	70		100	6.76

[a] Approximate, from n.m.r. integrals.



Scheme 1

workers,[3] that cyclisation of thiophen (1) with polyphosphoric acid (PPA) at 60 °C gave, after crystallisation of the hemi-oxalate, a 57% yield of the thienopyridine (2; R=H). However, we have been able to confirm the identity of the product using their conditions and to show that the crude cyclisation product contained no rearranged material detectable by ^{1}H n.m.r. spectroscopy. Further, with polyphosphoric acid, the methyl analogue (1; R=Me), in which rearrangement is even more likely, gave only the thieno[2,3-*c*]pyridine (2; R=Me).

It is possible that the formation of different products is related to different mechanisms of cyclisation, one in the strongly acidic trifluoroacetic acid involving a carbonium ion intermediate and the other in the much less strongly acidic polyphosphoric acid involving a complexed alcohol (Scheme 1).

Scheme 2. Only one optical form is represented for simplicity.

Hydrogen fluoride, which is also relatively weakly acidic, gave a similar result to polyphosphoric acid (Table 1).

If cyclisation of the complexed alcohol to give the *spiro*-intermediate is unfavourable for steric reasons, as seems quite probable from molecular models, then the lack of rearrangement in polyphosphoric acid and hydrogen fluoride is explained. Cyclisation of a complexed alcohol, in a manner analogous to an S_N2 reaction, is also consistent with the observation by Maffrand and co-workers[3] of inversion of configuration at the carbon, which is subject to nucleophilic attack, in *N*-thienylmethylnorephedrine (Scheme 2) when using polyphosphoric acid. It should be noted, however, that cyclisation of *N*-benzoylephedrines and pseudoephedrines in sulphuric acid, to give isoquinoline derivatives, produced the same *trans* product whether the starting material was *threo* or *erythro*.[4] This implies a mechanism analogous to an S_N1 reaction, sterically controlled by the adjacent chiral centre.

In the present case, formation of a carbonium ion would result in a less sterically hindered electrophile, which could be attacked by the thiophen ring from its electronically preferred 2-position to give the *spiro*-intermediate (Scheme 1), with subsequent ring-opening and reclosure to give the isomer (3). If these mechanistic arguments are correct, the presence of substituents on the phenyl ring which stabilise the carbonium ion will tend to favour rearrangement, whether the cyclising agent is strongly or weakly acidic. Some recent structural assignments for *p*-methoxyphenyl analogues[5] may therefore need to be revised, as indeed may some of the structures described in the earlier patent.[5]

We thank the S.E.R.C. for a C.A.S.E. award (to C. M.), Dr. C. W. Thornber for his interest, and I.C.I. Pharmaceuticals Division for additional support.

Received, 3rd February 1982; Com. 114

References

1 R. D. Waigh, *J. Chem. Soc., Chem. Commun.*, 1980, 1164, and references cited therein; C. Mackay and R. D. Waigh, manuscript in preparation.
2 C. Schneider, K. H. Weber, A. Langbein, W. D. Bechtel, and K. Boeke, G. P. 2 746 443 (1979), *Chem. Abs.*, 1979, **91**, 56 978x, B. P. 2 007 219 A, 1979.
3 J. P. Maffrand, R. Boigegrain, J. Courregelongue, G. Ferrand, and D. Frehel, *J. Heterocycl. Chem.*, 1981, **18**, 727.
4 D. L. Trepanier and S. Sunder, *J. Med. Chem.*, 1973, **16**, 342.
5 M. L. Brenner, Eur. P. Appl., 81 302 247.2, 1981.

Chemistry and Industry, 4 April 1983

Contributions should have novelty and must be brief. Manuscripts must be submitted in accordance with the instructions to authors, which were published in the 17 January 1983 issue (p 81). Authors are requested to note that manuscripts which do not accord with these instructions are currently being returned without consideration; in the case of overseas contributions return is by *seamail*. In order to expedite publication of accepted manuscripts, proofs are not circulated.

Rearrangement of *N*-(4-methoxybenzyl)-1-phenyl-2-aminoethanol to give 4-aryltetrahydroisoquinolines and a diarylethylamine

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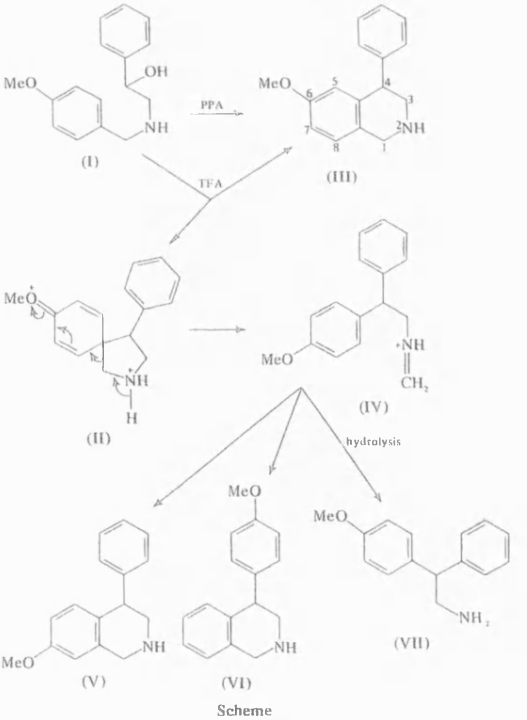
Over the past decade considerable use has been made of the cyclisation of *N*-benzylphenylethanolamines (see Scheme) in order to produce tetrahydroisoquinolines[1-6] of medicinal interest. The amino-alcohols have been cyclised with various reagents, for example polyphosphoric acid,[6,7] sulphuric acid,[1,5,6] hydrogen bromide,[7] methanesulphonic acid,[6] anhydrous aluminium chloride,[3,4] hydrochloric acid,[8] boron trifluoride,[6] and trifluoroacetic acid/sulphuric acid mixtures.[2] The general impression is that most acids are effective and that the choice is an individual one.

Recently, it has been found that the closely analogous *N*-(2-hydroxy-2-phenethyl)-2-aminomethylthiophens,[9] yield 4-phenyl-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridines in polyphosphoric acid (PPA) and 7-phenyl-4,5,6,7-tetrahydrothieno[3,2-*c*]pyridines in trifluoroacetic acid (TFA) by rearrangement through a *spiro*-intermediate. This observation prompted the authors to investigate the cyclisation of *N*-(4-methoxybenzyl)-1-phenyl-2-aminoethanol (I), in which the methoxy group activates the benzene ring towards formation of *spiro*-intermediate (II) (see Scheme).

Cyclisation in PPA (1h, 75-80°C), as indicated by ^{1}H n.m.r. and g.c., yielded only the unrearranged 6-methoxy-4-phenyl-1,2,3,4-tetrahydroisoquinoline (III, 96 per cent, mp HCl 237-8°C, Lit.[10] mp 238-9°C). By comparison, cyclisation in TFA (16h, reflux and then r.t. for 32h) was found to yield an oil (88 per cent) which from g.c. on 1 per cent Dexsil 300 at 210°C contained at least three products, of retention times and proportions 3.0 min (67 per cent), 4.4 min (21 per cent) and 5.3 min (12 per cent). The unrearranged product (III) from PPA cyclisation had a retention time of 4.4 min and an identical mass spectrum to that in the g.c./m.s. of the mixture, for the peak at 4.4 min.

If the *spiro*-intermediate (II) was formed, it would ring-open to yield the iminium ion (IV) which could be attacked by either of the aromatic rings giving the tetrahydroisoquinolines (V) and (VI) (see Scheme). Compounds (V), mp HCl 201-6°C, (Lit. mp[11] 210-11°C) and (VI), mp 74°C, (Lit. mp[12] 76°C) were unambiguously prepared by cyclisations of the corresponding amino-alcohols in PPA. The cyclisation of the amino-alcohol which gave the 7-methoxy-4-phenyl-1,2,3,4-tetrahydroisoquinoline (V) also yielded *ortho*-cyclised 5-methoxy-4-phenyl-1,2,3,4-tetrahydroisoquinoline. In this synthesis, *N*-(3-methoxybenzyl)-1-phenyl-2-aminoethanol was heated in PPA at 75-80°C for 1h to give a mixture of the two isomers with a crude yield of 94 per cent.



Scheme

The product hydrochlorides were separated by fractional crystallisation from absolute ethanol to give isomer (V) in 31 per cent isolated yield and the 5-methoxy analogue, mp 96-7°C, mp HCl 227-31°C, in 15 per cent isolated yield; the crude yield was 50 per cent of isomer (V) and 44 per cent of the 5-methoxy analogue, based on n.m.r. spectra and g.c. of the mixture.

Unfortunately both compounds (V) and (VI) had retention times of 5.3 min. Attempted separation by methylation and silylation failed to resolve the compounds on the above column and also on 3 per cent Dexsil 410 and 2 per cent OV-17 columns. Normal phase h.p.l.c. using a Partisil 5 column also failed to resolve the compounds. The g.c./m.s. of the peak at 5.3 min when compared to the m.s. of compounds (V) and (VI) indicated that both were present, but the exact proportions could not be determined.

The major product from treatment with TFA was the diarylethylamine (VII) arising from hydrolysis of the imine (IV). Compound (VII), mp HCl 176-8°C (Lit. mp[13] 182-4°C) was unambiguously prepared by the reduction of 2-(4-methoxyphenyl)-2-phenylethanenitrile. The authentic material had the same retention time as the compound giving the peak at 3.0 min, and was identical as shown by infrared, n.m.r. and mass spectral measurements.

Cyclisation of the amino-ethanol (I) in a mixture of TFA, 80 per cent *w/w* sulphuric acid and dichloromethane, essentially as described by Pridgen and co-workers,[10] gave as major product the non-rearranged isoquinoline (III), confirming their results. However, the presence of the rearrangement products in a total yield of 26 per cent was demonstrated by g.c..

It seems highly probable that the reactive intermediate (IV) would cyclise in much higher yield if one of the aromatic rings bore a suitable activating substituent. This will be the subject of further study.

G.c. studies with 1 per cent Dexsil 300 were performed on a Pye-Unicam series 204 instrument with a 1.7M × 4mm Chromosorb W.H.P. mesh 80-100 column. All compounds gave mass spectra, and infrared and ^{1}H n.m.r. spectra which were in accord with the structures proposed. All melting points are corrected. The SERC is thanked for an award (to M.R. Euerby).

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References

1 Hoffman, L., Ehrhart, G., & Schmitt, K., *Arzneim-Forsch.*, 1971, **21**, 1045
2 Brenner, L.M., & Wardell, J.R., Jr, Eur. Pat. Appln. E.P., 0040956, 1981; *Chem. Abstr.*, **96**, 162552
3 Brenner, L.M., Eur. Pat. Appl. E.P., 0043729, 1982; *Chem. Abstr.*, **96**, 142724
4 Jacob, J.N., Nichols, D.E., Kohli, J.D., & Glock, D., *J. Med. Chem.*, 1981, **24**, 1013-5
5 Trepanier, D.L., & Sunder, S., *ibid*, 1973, **16**, 342-7
6 Bobowski, G., Gottlieb, J.M., & West, B., *J. Heterocycl. Chem.*, 1980, **17**, 1563-8
7 Schwan, T.J., Loughheed, G.S., & Burrows, S.E., *ibid*, 1974, **11**, 807-10
8 Kametani, T., Takahashi, K., & Loc, C.V., *Tetrahedron*, 1975, **31**, 235-8
9 Mackay, C., & Waigh, R.D., *J. Chem. Soc. Chem. Commun.*, 1982, 793-4
10 Pridgen, L.N., Killmer, L.B., & Webb, R.L., *J. Org. Chem.*, 1982, **47**, 1985-9
11 Grethe, G., & Uskokovic, M.R., US Pat. 3666763, 1972; *Chem. Abstr.*, **77**, 75145
12 Deshpande, V.N., & Nargund, K.S., *J. Karnatak. Univ.*, 1956, **1**, 15-18; *Chem. Abstr.*, **52**, 7320
13 Huisgen, R., & Ruchardt, C., *Justus Liebigs Ann. Chem.*, 1957, **601**, 1-21

Methylthio Activating Groups in the Synthesis of Isoquinolines

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Methylthio activating groups have been found to improve the yields in six different isoquinoline syntheses: in four cases the improvement was from zero, in the unactivated system, to between 54 and 94%.

Most of the widely used syntheses of isoquinolines proceed by acid-catalysed attack of a suitable functionalised *N*-substituted phenethylamine or benzylamine on the benzene ring of the amine. They are thus representative of a wider range of reactions proceeding by electrophilic attack on an aromatic system, many of which are dependent on the presence of suitably placed electron-donating substituents. In the absence of activating groups, reactions of the last kind either fail completely, at temperatures below the decomposition point of the starting material, or give relatively poor yields under forcing conditions.

The most widely used activating group in the synthesis of isoquinolines has been the methoxy group, which is difficult to remove after cyclisation and is therefore not desirable where the unsubstituted ring is required. Methylthio appeared to be a suitable alternative, which would lend itself to removal by reductive desulphurisation. The activating potential of methylthio in this kind of reaction has been established in the benzylaminonitrile rearrangement.[1]

It was an important preliminary to this work that we should find a convenient synthesis of 3-methylthiobenzaldehyde,[2] from which all the phenethylamines and benzylamines used as intermediates are derived. All the starting materials were prepared from this aldehyde by variations of the literature methods used for the methoxy analogues.[3,4]

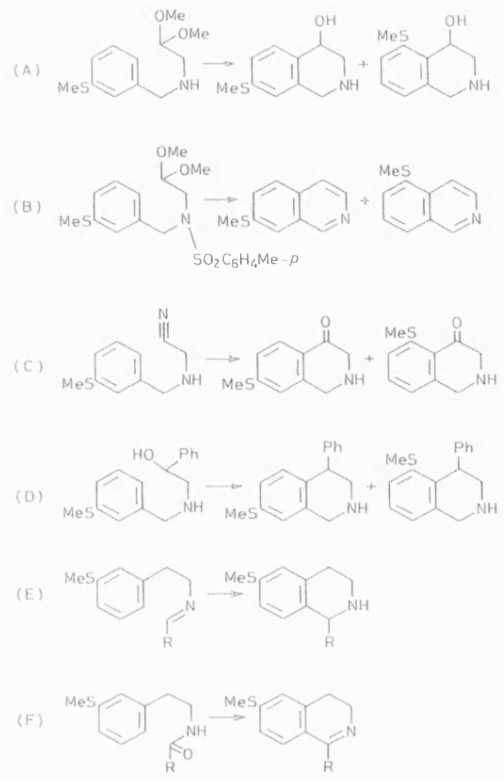
The types of cyclisation chosen for investigation were those which we have found to be reliable with the methoxy analogues: they are detailed in Scheme 1 as reactions (A)—(F). Where possible reaction conditions were as described in the original literature for the methoxy analogues; any variations or innovations are detailed in footnotes to Table 1. The use of anhydrous hydrogen fluoride in place of concentrated sulphuric acid for the benzylaminonitrile [reaction (C)] was particularly beneficial and is also useful for the methoxy analogue.[5]

It may be noted that, compared to the results in Table 1, reactions (A)—(C) and (E) do not give any cyclisation products[6—10] in the absence of an activating group. The

Table 1. The results and conditions for reactions (A)—(F).

			Isomer composition[g] (isolated yield)[h]/%			
Cyclisation[a]	Cyclising agent	Crude yield/%	*ortho*	M.p. (°C)	*para*	M.p. (°C)
(A)	HCl	91[b]	37 (7)	138—140	49 (32)	102
(B)	HCl	54	7 (4)	oil	47 (33)	46—47
(C)	H_2SO_4	13	7.5 (2[i])		5.5[i]	
	HF[c]	76	69 (51[i])	182—185[i]	7[i]	
(D)	PPA[d]	88	42 (6[i])	169—174[i]	46 (24[i])	203—208[i]
	TFA[e]	79	37		42	
	HF[f]	78	38		40	
(E) R = 1-Ph	TFA	94	—		94 (75)	79—80
R = 1-H	HCl	58	—		58 (51[i])	230—235[i]
(F) R = 1-Ph	$POCl_3$	89	—		89 (64[k])	166—169[k]
R = 1-H	$POCl_3$	21	—		21 (15[k])	147—151[k]

[a] For the methoxy analogues of (A), see refs. 6, 7; (B), ref. 8; (C), refs. 9, 10; (D), ref. 13; (E), ref. 14 (Ph), 15 (H); (F), ref. 16 (Ph), 17 (H). [b] 4-Hydroxytetrahydroisoquinolines (ref. 6). [c] Room temp., 20 days. [d] PPA = polyphosphoric acid, 75—80°C for 1 h. [e] TFA = trifluoro acetic acid, reflux, 7 h. [f] Room temp., 18 h. [g] Determined by gas chromatography or ^{1}H n.m.r. spectroscopy. [h] For identification purposes only: no attempt was made to optimise isolation procedures. [i] Identified by reduction and comparison with the product from reaction (A). [j] Hydrochloride. [k] Hemioxalate.



Scheme 1

Pictet–Spengler reaction [(E), R = Ph] was recently reported to succeed without activation,[11] but in our hands only starting materials were obtained.

We have desulphurised several of our cyclic products by treatment with nickel boride in yields of between 84 and 93%. The method, which is basically that of Truce and co-workers,[12] also reduces ketones to alcohols. In the present series of compounds the crude product from reaction (D), *i.e.* the mixture of methylthio-4-phenyltetrahydroisoquinolines, gave clean 4-phenyl-1,2,3,4-tetrahydroisoquinoline in 84% yield, using a molar ratio of organosulphur compound : nickel chloride : sodium borohydride of 1 : 10 : 30. Provided that sufficient nickel boride was formed the ratios were not critical. In some cases a basic product was adsorbed on the catalyst and was best liberated by dissolution in dilute hydrochloric acid followed by basification with ammonia to form the nickel complex before extraction with chloroform. With this modification applied when required, no desulphurisation has as yet failed. Some of the other compounds which have been desulphurised are as follows (product and percentage yield in brackets): 4-methylthiobenzaldehyde (benzyl alcohol, 94%), *N*-methyl-4-methylthiobenzylamine (*N*-methylbenzylamine, 91%), 3-(4-ethylthiobenzoyl)-3-methyl-1,2,3,4-tetrahydroisoquinoline[1] [3-(α-hydroxybenzyl)-3-methyl-1,2,3,4-tetrahydroisoquinoline, 91%], 4-(4-methylthiophenyl)-3-imidazoline-5-spirocyclohexane[1] (5-phenylimidazoline-4-spirocyclohexane, 92%), 4-(4-methylthiophenyl)-1,5,5-trimethyl-3-imidazoline[1] (5-phenyl-3,4,4-trimethylimidazoline, 93%). The imidazolines underwent a double-bond shift which will be discussed elsewhere.

Since the activating group is removed later it does not matter whether cyclisation proceeds *ortho* or *para*, but it is interesting to note the variation in the proportions of the two isomers. The benzylamines gave proportionately more material from cyclisation *ortho* to the activating group than the phenethylamines: in one case the *ortho* product [reaction (C) with hydrogen fluoride] predominated.

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References

1 M. R. Euerby and R. D. Waigh, *J. Chem. Res.*, 1982, (*S*) 240; (*M*) 2416.
2 M. R. Euerby and R. D. Waigh, *Synth. Commun.*, 1981, **11**, 849.
3 J. M. Gulland and C. J. Virden, *J. Chem. Soc.*, 1929, 1791.
4 T. Kametani, K. Kigasawa, M. Hiiragi, and H. Ishimaru, *J. Chem. Soc. C*, 1971, 2632.
5 M. R. Euerby and R. D. Waigh, unpublished work.
6 J. M. Bobbitt, J. M. Kiely, K. L. Khanna, and R. Eberman, *J. Org. Chem.*, 1965, **30**, 2247.
7 J. M. Bobbitt and J. C. Sih, *J. Org. Chem.*, 1968, **32**, 856.
8 A. J. Birch, A. H. Jackson, and P. V. R. Shannon, *J. Chem. Soc., Perkin Trans. 1*, 1974, 2185.
9 D. N. Harcourt, N. Taylor, and R. D. Waigh, *J. Chem. Soc., Perkin Trans. 1*, 1978, 722.
10 D. N. Harcourt and R. D. Waigh, *J. Chem. Soc. C*, 1971, 967.
11 P. Kumar, K. N. Dhawan, K. Kishor, K. P. Bhargava, and R. K. Satsangi, *J. Heterocycl. Chem.*, 1982, **19**, 677.
12 W. E. Truce and F. M. Perry, *J. Org. Chem.*, 1965, **30**, 1316.
13 T. J. Schwan, G. S. Lougheed, and S. E. Burrows, *J. Heterocycl Chem.*, 1974, **11**, 807.
14 R. Paul, J. A. Coppola, and E. Cohen, *J. Med. Chem.*, 1972, **15**, 720.
15 L. Helfer, *Helv. Chim. Acta*, 1924, **7**, 945.
16 S. Ishiwata and K. Itakura, *Chem. Pharm. Bull.*, 1968, **16**, 778.
17 H. Corrodi and G. Jonsson, *Helv. Chim. Acta*, 1966, **49**, 798.

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A NEW SYNTHESIS OF 1,2-DIHYDRO-β-CARBOLIN-4(3H)-ONES

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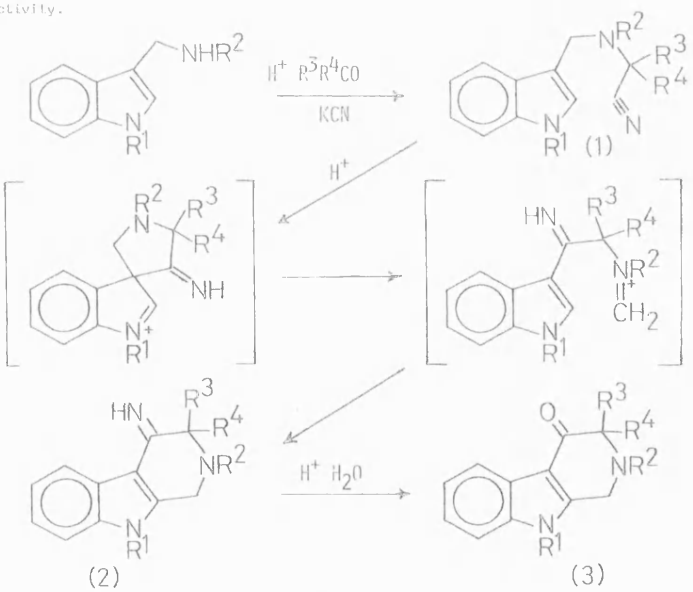
Abstract — Aminonitriles derived from 3-aminomethylindoles are rearranged by treatment with polyphosphoric acid at 80°C or sulphuric acid at 0°C, to give 1,2-dihydro-β-carbolin-4(3H)-ones.

It has been well established that cyclisation of benzylaminonitriles to isoquinolinones[1-5] and other products[6] involves the rearrangement of a spiro intermediate. Rearrangement of spiro intermediates derived from 3-substituted indoles in the formation of tetrahydrocarbazoles has been the subject of intensive study by Jackson[7-11] and co-workers. Since 3-aminomethylindoles (skatylamines) are readily available, rearrangement of a skatylaminoacetonitrile (Scheme) offers an attractive route to new β-carbolines, currently of considerable interest for their C.N.S. activity.



SCHEME

Aminonitriles (1) were prepared by Strecker reactions on 3-aminomethylindoles obtained via the Vilsmeier-Haack formylation of indole and reduction of the corresponding oxime or imine. Cyclohexanone and acetone were chosen for the Strecker reactions, since these had given good yields in the isoquinolinone syntheses[1]. No identifiable product was obtained when formaldehyde was used as the carbonyl component of the mixture.

Table 1 describes the yields, melting points and substitution patterns of the aminonitriles prepared. Structures were confirmed by IR, NMR and MS, and for solids by C,H,N analysis.

Table 1 Aminonitriles (1)

	R^1	R^2	R^3	R^4	Yield (%)	M.pt (^oC)
a	H	H	$-(CH_2)_5-$		88	104-5
b	H	H	CH_3	CH_3	75	72-4
c	CH_3	H	$-(CH_2)_5-$		74	57-8
d	CH_3	H	CH_3	CH_3	97	92-3
e	CH_3	CH_3	$-(CH_2)_5-$		88	116-7

As cyclising agent we initially tried sulphuric acid under the conditions used to rearrange benzylaminoacetonitriles[1-6] but observed extensive polymerisation and/or sulphonation; no identifiable products were isolated. Polyphosphoric acid (80^{o}C, 1h) was more successful, as described in Table 2, but reaction conditions were critical; yields decreased rapidly at temperatures above 80^{o}C. Sulphuric acid at lower temperatures (0^{o}C for 15 min) was a considerable improvement (Table 2), in the five instances for which we have data.

Table 2 β-Carbolinones (3)

Product	Yield (%)†	Melting Point (^oC)	>C=O (cm^{-1})
a	25 (88)*	189	1610
b	20 (50)	196-8	1630
c	24 (41)	184-6	1630
d	34 (65)	184-6	1630
e	17 (53)	120-2	1605

* Isolated as imine (2), mp 206-8^o, after allowing the reaction mixture to stand for only 4 h at room temperature.

† Figures in brackets are yields from reactions in cold sulphuric acid (see text).

Both with polyphosphoric acid and sulphuric acid, the reaction mixture was cooled if necessary, diluted, and allowed to stand, filtered to remove polymeric by-products, and basified. The product was filtered off or extracted with chloroform or ethyl acetate. It was found that hydrolysis of the imines (2) to the ketones (3) was slow compared with iminotetrahydroisoquinolines[1]; work-up before 24h after dilution tended to give (2) rather than (3), or mixtures. The imines that were isolated were also converted with aqueous acid to the ketones as confirmation of structure.

All the products have been characterised by IR, NMR, MS and most by C,H,N analysis. The only question concerning the carbolines was the position of attachment of the carbonyl group, at C_2 or C_3 of the indole i.e. whether rearrangement had in fact occurred. In this respect the work of Neukomm and Hesse[12] was particularly helpful, allowing a comparison with directly analogous oxocarbazoles. Of major significance are (a) the carbonyl stretching frequency, which for 3-acyl indoles is < 1630 cm^{-1}, whereas for 2-acyl indoles it is 30-50 cm^{-1} higher (b) the downfield shift in the ^1H NMR spectrum of the benzene-ring proton near to the carbonyl group; in 2-acyl indoles there is no such proton (c) the UV spectra, which show three bands of similar wavelength and intensity to the 4-oxocarbazoles.

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REFERENCES

1. D.N. Harcourt and R.D. Waigh, J. Chem. Soc. (C), 1971, 967.
2. D.N. Harcourt, N. Taylor and R.D. Waigh, J.C.S. Perkin 1, 1978, 722.
3. D.N. Harcourt, N. Taylor and R.D. Waigh, J.C.S. Perkin 1, 1978, 1330.
4. D.N. Harcourt, N. Taylor and R.D. Waigh, J. Chem. Research (M), 1978, 1954.
5. D.N. Harcourt, N. Taylor and R.D. Waigh, J.C.S. Chem. Comm., 1972, 644.
6. R.D. Waigh, J.C.S. Chem. Comm., 1980, 1164.
7. A.H. Jackson, B. Naidoo and P. Smith, Tetrahedron, 1968, 24, 6119.
8. K.M. Biswas and A.H. Jackson, Tetrahedron, 1969, 25, 227.
9. R. Iyer, A.H. Jackson, B. Naidoo and P.V.R. Shannon, J.C.S. Perkin 2, 1973, 872.
10. R. Iyer, A.H. Jackson and P.V.R. Shannon, J.C.S. Perkin 2, 1979, 878.
11. J.S.L. Ibaceta-Lizana, R. Iyer, A.H. Jackson and P.V.R. Shannon, J.C.S. Perkin 2, 1978, 733.
12. G. Neukomm and M. Hesse, Helv. Chim. Acta, 1977, 60, 2431.

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A Theoretical Study of the Site Selectivity of Activating Methylthio and Methoxy Groups in the Synthesis of Isoquinolines

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The site selectivity of activating methylthio and methoxy groups in the synthesis of isoquinolines has been studied theoretically in terms of the static index approach. The dependence of the relative amounts of *ortho*- and *para*-isomer produced upon the nature of the electrophilic group has been explained in terms of the degree of charge localization in the electrophile, together with the calculated electronic structure of the activated benzene ring.

Most of the widely used syntheses of isoquinolines proceed *via* acid-catalysed attack of a suitable functionalized *N*-substituted phenethylamine or benzylamine on the benzene ring of the amine. To facilitate the attack of the electrophilic function of the side-chain upon the benzene ring, the latter is activated by suitable electron-donating substituents. In the absence of such activating groups, the reaction either fails, or gives poor yields. The most widely used activating group has been methoxy, but this is difficult to remove after cyclization and is thus to be avoided when the unsubstituted ring is sought. However, methylthio has been found to be a suitable alternative,[1] which may be subsequently removed by reductive desulphurization. With the MeS group placed *meta* to the attacking side-chain, activation of the positions *ortho* and *para* to this group can yield two possible isomeric final products. The reactions we have studied are detailed in the Scheme; the first product is referred to as the *ortho*-isomer and the second as the *para*-isomer. The charged structures depicted in the Scheme are simplistic, taking no account of charge delocalization or solvent participation. However, we believe them to represent the major features of the reactive state. We have found that the relative amounts of the *ortho*- and *para*-products are dependent upon the nature of the side-chain electrophile.[2] A summary of these experimental results, mainly for methylthio activation, together with some for methoxy activation, is given in the Table. (Reaction conditions for the methoxy analogues were as described for the methylthio compounds.[2] The product ratios for reaction C were derived from cyclizations in anhydrous hydrogen fluoride.[2]) It can be seen that the products range from essentially 100% *ortho*-isomer (for a carbocation electrophile), to 100% *para*-isomer (for an iminium ion electrophile). In this paper we seek a theoretical explanation of these findings.

Table. Isomer composition of activating MeS and MeO groups

	MeS			MeO		
	Isomer composition [Yield (%)]			Isomer composition [Yield (%)]		
Reaction	*ortho*	*para*	*ortho*/*para*	*ortho*	*para*	*ortho*/*para*
A	95					
B	74					
C	69	7	10	59	41	1.4
D	49	37	1.3	10	58	0.2
E	40	40	1.0	45	53	0.8
F	8	53	0.2			
G	7	47	0.1	4	71	0.06
H		21			25	
I		58			68	
J		89		7.5	78.5	0.1
K		94		2	85	0.03

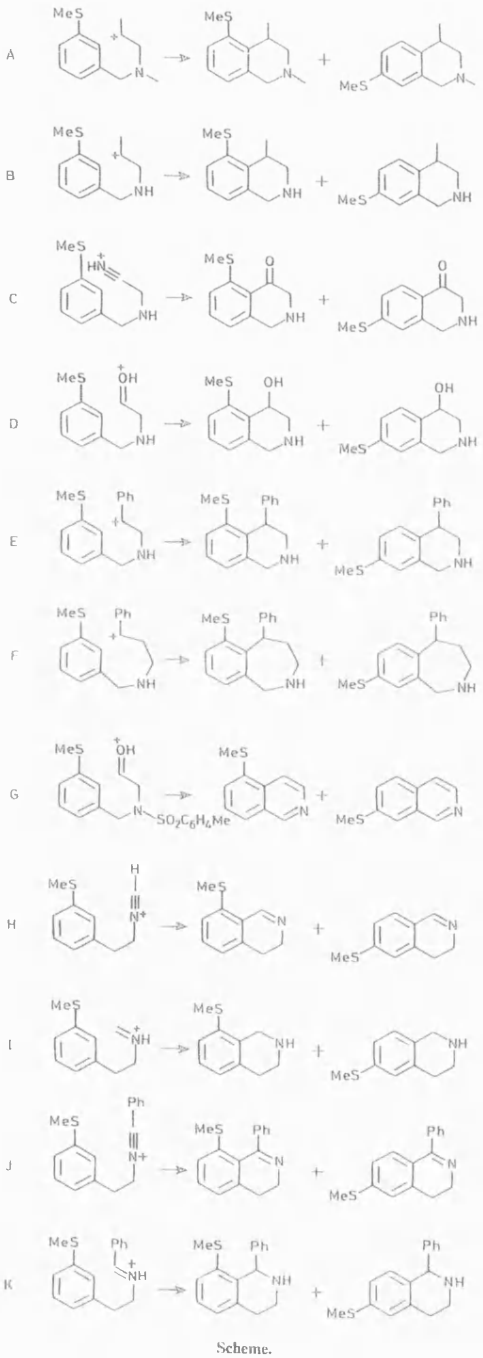
Theoretical Methods

We shall consider electronic and steric effects, both of which may influence the relative amounts of *ortho*- and *para*-isomer produced. The simplest theoretical approach which accounts for electronic effects is to attempt to correlate the observed reactivities with the ground-state electronic structures of the reactants, using electronic properties ('static indices'), which are taken to be indicative of important factors influencing the transition state.[3,4] Such simple considerations are appropriate in the absence of accurate calculations of the transition state, which are certainly not feasible at the present time if *ab initio* wavefunctions are employed.

We examine the possibilities that the reactions considered may be dominated by 'frontier orbital'[4] or 'charge-control'[3] considerations. Attack on the benzene ring by a 'hard' electrophile, in which the positive charge is localized, would be expected to be 'charge-controlled,' and in that case the preferred site of attack may be expected to correlate with the charge distribution of the methoxy- or methylthio-substituted benzene ring.

In the 'frontier orbital' approach, attention is focused on the HOMO of the nucleophile (the benzene ring) and the LUMO of the electrophile, the stability of the transition state being taken to depend on the amplitudes of these two MOs on the atoms involved in bond formation.[4,5] Such an approach is generally taken to be appropriate for attack by a 'soft' electrophile, in which the positive charge is delocalized.[4]

To examine both the charge distribution and form of the HOMO of the nucleophile, *ab initio* SCF–MO calculations were carried out for 3-methyl(thioanisole) and 3-methylanisole. The geometries of these two molecules were estimated as follows. Data for 3-methylanisole were taken from published electron diffraction results for anisole;[6] a methyl group in a standard geometry[7] was added to the 3-position to take some account of the side-chain of the benzene ring. In both molecules the methyl groups attached to the heteroatom are assumed to be coplanar with the benzene ring. Bond lengths and angles for the PhS fragment of 3-methyl(thioanisole) were taken from those quoted in a microwave study of benzenethiol;[8] the CH_3S group geometry was adapted from that of dimethyl sulphide.[9] As for anisole, a methyl group was added at the 3-position to model the side-chain. Although this side-chain is different for the



Scheme.

various reactions, such differences are not expected to alter the electronic structure of the benzene ring so as to alter significantly the conclusions of this study. The SCF-MO calculations were carried out in an STO-3G basis.[10]

Theoretical Results

The calculated charge distributions obtained by a Mulliken population analysis, and HOMO coefficients are given in the Figure. From these results we conclude that (*a*) a 'charge-controlled' mechanism favours the *ortho*- over the *para*-isomer, since the corresponding formal atomic charges at the *ortho*- and *para*-positions are −0.094 and −0.080 for 3-methyl(thioanisole) and −0.095 and −0.086 for 3-methylanisole; (*b*) a 'frontier orbital' mechanism favours the *para*- over the *ortho*-isomer since the magnitudes of the HOMO coefficients are 0.385 and 0.248 at the *para*- and *ortho*-positions for 3-methyl(thioanisole) and 0.493 and 0.309 for 3-methylanisole. Thus, from these quite simple theoretical considerations we would expect that cyclization involving a 'hard' electrophile would favour the *ortho*-isomer, whilst a 'soft' electrophile would favour the *para*-isomer.

The electrophiles in the Scheme can be divided into three main classes: carbocations, in which the charge resides formally on a carbon atom; protonated carbonyls in which the charge lies on an oxygen atom; and protonated imines and isonitriles in which the charge resides, at least formally, on a nitrogen atom. Electronegativity considerations suggest that the positive charge on the electrophilic carbon atom will be least for the third group, somewhat greater for the second group, and greatest for the carbocations. Taking the degree of positive charge localization to determine the 'hardness' of the electrophile leads to the prediction that the carbocations will give the greatest *ortho*:*para* ratios, the carbonyls rather lower ratios, and the imines and isonitriles the lowest ratios of all. This is the generally observed trend from the data in the Table.

There are however exceptions to these simple rules, evident from the data in the Table, which we now discuss. Groups attached to the reactive carbon atom of the electrophile may exert an influence on the 'hardness'. In particular, a phenyl group will partially delocalize the positive charge, leading to a decrease in 'hardness,' and to a predicted lowering of the *ortho*:*para* ratio. This is indeed found experimentally for the reactions E and F as compared with A and B. However, the reaction F leads to considerably less *ortho*-isomer than does E, in spite of very similar electronic effects. Large groups attached to the electrophilic carbon atom may be expected to favour the *para*-isomer because of steric interactions with the XMe group. The difference observed between E and F may possibly be

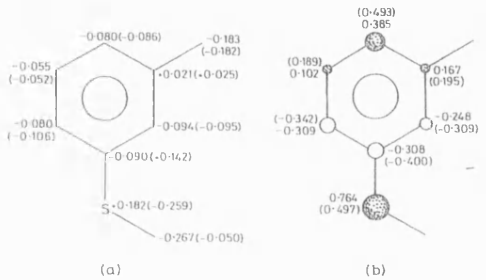


Figure. Electronic structure of 3-methyl(thioanisole) and 3-methylanisole. (a) Formal atomic charges. (b) Coefficients of the HOMO. The values for 3-methylanisole are given in parentheses

attributed to the increased steric repulsion on formation of the seven- as opposed to the six-membered ring on cyclization. However, the observed differences in *ortho*:*para* ratio between D and G cannot be explained by our simple considerations. Furthermore, C unexpectedly appears from the *ortho*:*para* ratios to involve a harder electrophile than the isonitriles.

Turning now to the data available for methoxy, as opposed to methylthio activation (Table), we note that the trends discussed for the latter group are also present here. However, the *ortho*:*para* ratio is in most cases smaller than for the corresponding methylthio-activated reaction. The calculation for 3-methylanisole reveals *smaller* charge differences between the *ortho*- and *para*-positions than for 3-methyl(thioanisole) (Figure). Hence, a 'charge-controlled' mechanism still favours production of the *ortho*-isomer, but not to such an extent as in the methylthio case. This effect is reinforced in the 'frontier orbital' mechanism since in the HOMO of 3-methylanisole (Figure) there is a *larger* difference between the magnitudes of the coefficients at the *ortho*- and *para*-positions than in the case of 3-methyl(thioanisole). Thus, the smaller *ortho*:*para* ratio observed for methoxy activation may be attributed to these two effects.

Conclusions

We have used the static index approach to understand the site selectivity of activating groups in the synthesis of isoquinolines. We find that considerations of the degree of charge delocalization in the electrophile, together with calculations of the electronic structure of the nucleophile, are broadly able to account for the observed trends in the isomer product ratios in these cyclization reactions. However, the role of steric effects in such reactions has yet to be more clearly defined.

References

1 M. R. Euerby and R. D. Waigh, *J. Chem. Res.*, 1982, (*S*) 240; (*M*) 2416.
2 M. R. Euerby and R. D. Waigh, *J. Chem. Soc., Chem. Commun.*, 1984, 127.
3 G. Klopman, *J. Am. Chem. Soc.*, 1968, **90**, 223.
4 I. Fleming, 'Frontier Orbitals and Organic Chemical Reactions,' Wiley, London, 1976.
5 G. Klopman and R. F. Hudson, *Theor. Chim. Acta*, 1967, **8**, 165.
6 H. M. Seip and R. Seip, *Acta Chem. Scand.*, 1973, **27**, 4024.
7 J. A. Pople and M. Gordon, *J. Am. Chem. Soc.*, 1967, **89**, 4253.
8 K. I. Johansson, H. Oldeberg, and H. Selen, *Arkiv Fysik*, 1966, **33**, 313.
9 L. Pierce and M. Hayashi, *J. Chem. Phys.*, 1961, **35**, 479.
10 W. J. Hehre, R. F. Stewart, and J. A. Pople, *J. Chem. Phys.*, 1969, **51**, 2657.

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SYNTHESIS OF 4,5-DIHYDRO-1H-IMIDAZOLES FROM 1,5-DIHYDRO-2H-IMIDAZOLES USING 'NICKEL BORIDE'

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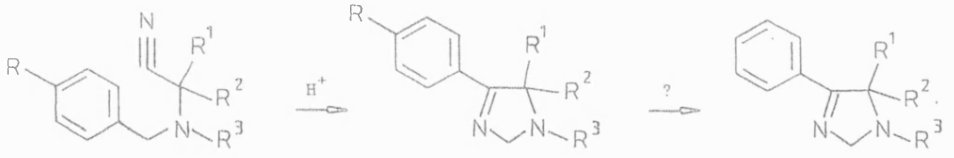
Abstract - In situ prepared 'nickel boride' generated from sodium borohydride and nickel (II) chloride cleanly transforms 1,5-dihydro-2H-imidazoles into 4,5-dihydro-1H-imidazoles in high yields by a double bond migration.

Research into the use of methylthio substituents as removable activating groups in the synthesis of N-heterocycles[1,2] prompted their application to the synthesis of 1,5-dihydro-2H-imidazoles (2) by the cyclisation of the methylthio activated benzylaminonitriles (1)[3] (Scheme 1). In the absence of the methylthio activating group, cyclisation fails to occur,[3] but it was anticipated that the unsubstituted 4-phenyl-1,5-dihydro-2H-imidazole (3) could be obtained by removal of the methylthio group from (2). This could be effected by reductive desulphurisation using 'nickel boride' generated by the action of sodium borohydride on nickel (II) chloride in situ, a method which we have shown to be superior in many respects to Raney nickel.[4]

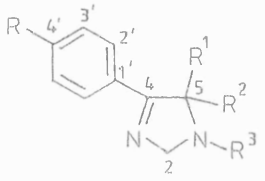


(1) (2) (3)

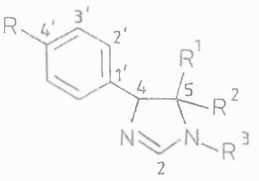
(Scheme 1, R = MeS)

As expected, the methylthio substituted 1,5-dihydro-2H-imidazoles (2a and b), when treated with 'nickel boride', underwent reductive desulphurisation of the thioether bond; unexpectedly, migration of the dihydroimidazole double bond occurred yielding the corresponding 4,5-dihydro-1H-imidazoles (4a and b) which are of potential pharmacological interest.[5] As a corollary the

analogous methoxy substituted 1,5-dihydro-2H-imidazoles (2c and d) were treated with 'nickel boride'. In these reactions double bond migration occurred yielding the corresponding methoxy substituted 4,5-dihydro-1H-imidazoles (4c and d). (Scheme 2).



'Nickel boride'



(2a - d)

	R	R^1	R^2	R^3
a	MeS	$(CH_2)_5$		H
b	MeS	Me	Me	Me
c	MeO	$(CH_2)_5$		H
d	MeO	$(CH_2)_5$		Me

(4a - d)

	R	R^1	R^2	R^3
a	H	$(CH_2)_5$		H
b	H	Me	Me	Me
c	MeO	$(CH_2)_5$		H
d	MeO	$(CH_2)_5$		Me

(Scheme 2)

The structure of the 4,5-dihydro-1H-imidazoles (4) was unambiguously confirmed by the use of ^1H and ^{13}C n.m.r. and mass spectroscopy. The ^1H n.m.r. spectra of the 4,5-dihydro-1H-imidazoles (4) exhibited characteristic features including resonances in the region of δ_H 7.22 - 6.97 and 4.82 - 4.49 for the amidine and methine protons at positions 2 and 4 respectively; in comparison, the 1,5-dihydro-2H-imidazoles (2) possessed resonances in the region of δ_H 4.79 - 4.58 for the methylene protons at position 2.[3,6] Off resonance ^{13}C n.m.r. spectroscopy easily distinguished between the 1,5-dihydro-2H-imidazoles (2) and the 4,5-dihydro-1H-imidazoles (4) (Table 1): the former exhibited singlets and triplets in the region of δ_C 176.5 - 175.7 and 82.1 - 73.9 for the carbons in positions 4 and 2 respectively, whereas the latter possessed two doublets in the region of δ_C 78.5 - 73.5 and 156.7 - 152.2 for the carbons in the same positions. Carbon assignments (Table 1) were completed using the D.E.P.T. technique.

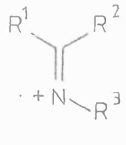
The plane of symmetry in the 1,5-dihydro-2H-imidazoles (2) is absent in the 4,5-dihydro-1H-imidazoles (4) and as a consequence the R^1 and R^2 substituents in the latter are non-equivalent. This difference in symmetry is observable in both the ^1H and ^{13}C n.m.r. spectra.

The fragmentation pattern in the mass spectra is also diagnostic in that the base peaks from the 1,5-dihydro-2H-imidazoles (2) correspond to the aziridinium ion (5),[3,6] whereas the base peaks for the 4,5-dihydro-1H-imidazoles (4) correspond to the iminium ion (6).

Table 1: ^{13}C N.m.r. spectra of the dihydroimidazoles (2a - d and 4a - d), (Solvent $CDCl_3$).

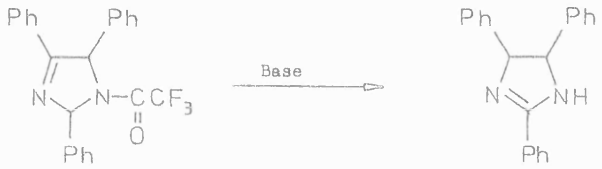
Compound	R	R^1	R^2	R^3	C-2	C-4	C-5	C-1'	C-2'	C-3'	C-4'
2a	14.5 (q)	32.5 25.0	25.0 (3 x t)	-	73.9 (t)	176.0 (s)	71.0 (s)	129.6 (s)	124.9 (d)	127.7 (d)	140.2 (s)
2b	14.7 (q)	20.5 (q)	20.5 (q)	30.7 (q)	79.4 (t)	176.1 (s)	67.0 (s)	129.1 (s)	125.0 (d)	127.4 (d)	140.9 (s)
2c	54.8 (q)	33.0 22.6	25.2 (3 x t)	-	73.9 (t)	175.7 (s)	71.3 (s)	125.4 (s)	113.0 (d)	128.7 (d)	160.0 (s)
2d	55.0 (q)	38.1 22.7	25.5 (3 x t)	29.6 (q)	82.1 (t)	176.5 (s)	74.0 (s)	127.0 (s)	113.5 (d)	129.2 (d)	160.5 (s)
4a	-	39.4 25.1 22.6	33.5 22.6 (5 x t)	-	153.5 (d)	73.5 (d)	68.0 (s)	138.2 (s)	127.9 (d)	127.3 (d)	127.3 (d)
4b	-	26.0 (q)	19.8 (q)	28.2 (q)	156.7 (d)	78.3 (d)	65.3 (s)	139.0 (s)	127.0 (d)	127.3 (d)	125.1 (d)
4c	55.1 (q)	39.4 25.5 22.7	33.8 23.0 (5 x t)	-	152.2 (d)	78.3 (d)	68.3 (s)	131.5 (s)	113.5 (d)	128.5 (d)	159.0 (s)
4d	55.1 (q)	32.6 25.1 21.8	29.3 22.9 (5 x t)	27.8 (q)	155.7 (d)	77.6 (d)	65.7 (s)	131.6 (s)	113.2 (d)	129.7 (d)	158.8 (s)

(5)



(6)

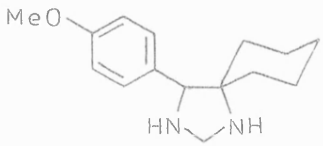
Double bond migration involving conversion of a 1,5-dihydro-2H-imidazole to a 4,5-dihydro-1H-imidazole was observed[7] when the 1,5-dihydro-1H-imidazole (7) was treated with base (Scheme 3).



(7)

(Scheme 3)

However, in our hands the 1,5-dihydro-2H-imidazole (2a) was inert to the effects of base treatment and also to nickel (II) chloride alone. In contrast, exposure of the 1,5-dihydro-2H-imidazole (2c) to sodium borohydride yielded the corresponding 1,3,4,5-tetrahydro-2H-imidazole (8) via reduction of the imine, suggesting that it is 'nickel boride' which is responsible for the double bond migration of 1,5-dihydro-2H-imidazoles (2) yielding 4,5-dihydro-1H-imidazoles (4).



(8)

Research is currently being undertaken to elucidate the mechanism, applicability and scope of this reaction.

EXPERIMENTAL

Melting points were determined on a Reichert hot stage apparatus and are corrected. I.r. spectra were recorded with a Pye-Unicam SP3-100 instrument for potassium bromide discs or liquid films. ^{1}H and ^{13}C n.m.r. spectra, unless otherwise stated, were recorded on a Bruker WP80 spectrometer operating at 80 and 20.1 MHz respectively. M.s. data were obtained using an A.E.I. M530 spectrometer using electron ionisation unless otherwise stated. Microanalysis was carried out on a Perkin-Elmer 240 CHN analyser. The methylthio and methoxy substituted 1,5-dihydro-2H-imidazoles (2a - d) were

prepared as described in the literature.[3,6]

General Reaction

The 1,5-dihydro-2H-imidazole (2, 2mmol) and nickel (II) chloride hexahydrate (12mmol) were dissolved in methanol (20ml) and sodium borohydride (60mmol) added portionwise over a period of 30 min to the ice-cold mixture. Evolution of hydrogen and a black precipitate were observed on addition of sodium borohydride. When the addition was complete, the ice-bath was removed and stirring continued for 16 - 18 h at room temperature. To the ice-cooled reaction mixture was added hydrochloric acid (32% $^{w}/_{w}$, 40ml) and stirring continued for 10 min. Then ammonia solution (s.g. 0.88, 60ml) was added slowly to basify the mixture. The reaction mixture was filtered through a bed of 'celite', the filter cake washed with chloroform (3 x 25ml) and this chloroform used to extract the product from the aqueous filtrate. The combined chloroform extracts were washed with brine (1 x 25ml), dried with magnesium sulphate and evaporated to yield the 4,5-dihydro-1H-imidazoles (4a - d). The 4,5-dihydro-1H-imidazoles could be purified by column chromatography using basic (grade 1) alumina eluting with varying proportions of chloroform and methanol or by crystallisation from chloroform - petroleum (60-80°C).

Table 2: Analytical data for the 4,5-dihydro-1H-imidazoles (4a - d).

Compound	Yield[1]	mp °C	Solvent	Found %			Required %		
				C	H	N	C	H	N
4a	92(57)	152-155	$CHCl_3$-petroleum 60-80°C	78.2	8.4	13.0	78.5	8.5	13.1
4b	93(60)	125-127[2]	EtOH-di-iso-propyl ether	60.5	6.7	10.0	60.4	6.5	10.1
4c	96(75)	142-144	$CHCl_3$-petroleum 60-80°C	73.8	8.2	11.2	73.8	8.2	11.5
4d	85(67)	oil[3]							

[1] Crude yields calculated from n.m.r. data, values in parenthesis are purified non-optimised yields

[2] Hemioxalate salt

[3] Salt forms too hygroscopic for analysis

Table 3: ^{1}H N.m.r. and i.r. data for the 4,5-dihydro-1H-imidazoles (4a - d).

Compound	ν max/cm^{-1} C = N	^{1}H N.m.r. (δ values in p.p.m. Solvent $CDCl_3$)
4a	1590	[1] 7.27(5H,s,ArH), 7.21(1H,s,N=CH-N), 4.49(1H,s,ArCHN), 4.28(1H,s, NH exchangeable), 1.84-0.40(10H,m,5xCH_2).
4b	1590	[1] 7.27(5H,s,ArH), 7.11(1H,d,J=2Hz,N=CH-N), 4.76(1H,d,J=2Hz,ArCHN), 2.78(3H,s,CH_3N), 1.37(3H,s,CH_3C), 0.62(3H,s,CH_3C).
4c	1585	7.33(1H,s,N=CH-N), 7.16(2H,d,J=8.5Hz,ArH), 6.85(2H,d,J=8.5Hz, ArH), 4.44(1H,s,ArCHN), 3.80(3H,s,CH_3O), 2.86(1H,s,NH exchangeable), 1.92-0.67(10H,m,5xCH_2).
4d	1590	7.13(2H,d,J=8.5Hz,ArH), 6.97(1H,s,N=CH-N), 6.81(2H,d,J=8.5Hz, ArH), 4.82(1H,s,ArCHN), 3.80(3H,s,CH_3O), 2.78(3H,s,CH_3N), 1.89-0.65(10H,m,5xCH_2).

[1] 60 MHz Perkin-Elmer R12B

Table 4: Mass spectral fragmentation of the 4,5-dihydro-1H-imidazoles (4a - d).

Compound	Principal fragments, m/z (rel. intensity %)
4a	m/z 214(M^+,44%), 132(12), 120(18), 106(68), 98(92), 83(100), 79(11), 67(10), 47(14), 30(5).
4b	m/z 188(M^+,20%), 173(2), 117(7), 90(8), 72(100), 56(5), 45(14), 29(3).
4c	m/z 244(M^+,33%), 136(60), 121(17), 109(57), 98(72), 67(56), 40(100).
4d	m/z 258(M^+,45%), 136(11), 121(24), 112(100), 91(16), 86(30), 81(22), 77(20), 68(25), 55(28), 44(100), 40(93).

Sodium borohydride reduction of 1,5-dihydro-2H-imidazole (1c)

The general method was followed with the exception that nickel (II) chloride hexahydrate was omitted. 1,3,4,5-Tetrahydro-2H-imidazole (8) was formed as a mobile brown oil which, after purification by column chromatography on silica gel 60 (Merck 70-230 mesh) eluting with varying proportions of methanol in ethyl acetate, gave a golden oil (79%), δ_H ($CDCl_3$) 7.16 and 6.84(2 x 2H, 2 x d, J=8.5Hz, AA'XX', ArH), 4.18 and 3.97(2H, 2 x d, J=9Hz, NCH_2N), 3.82(4H, s, CH_3O and CHN), 1.82(2H, s, NH x 2 exchangeable), 1.85-0.47(10H, m, CH_2 x 5); δ_C ($CDCl_3$) 158.7(C-4',s) , 133.0 (C-1',s), 128.6(C-3',d), 113.4(C-2',d), 70.8(C-4,d), 63.8(C-5,s), 62.2(C-2,t), 55.0(CH_3O,q), 36.2, 33.0, 25.8, 23.2, 22.5(CH_2x5); m/z (chemical ionisation, isobutane), 247(M^+,14%), 235(28), 218

(60), 148(7), 136(8), 98(100), 94(17), 81(5), 57(7). The picrate had m.p. 217-220°C (decomp.) (From EtOH-diethyl ether) (Found: C,46.0; H,4.3; N,15.9. $C_{27}H_{28}N_8O_{15}$ requires C,46.0; H,4.0; N,15.9%).

ACKNOWLEGEMENT

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REFERENCES

1 M.R.Euerby and R.D.Waigh, J.Chem.Soc.,Chem.Commun., 1984, 127.

2 M.R.Euerby, R.D.Waigh, I.H.Hillier, and J.P.Huke, J.Chem.Soc.,Perkin Trans.2, 1985, 1151.

3 M.R.Euerby and R.D.Waigh, J.Chem.Res., 1982, (S)240, (M)2417.

4 M.R.Euerby and R.D.Waigh, Syn.Commun., 1986, in the press.

5 K.Hoffman, 'The Chemistry of Heterocyclic Compounds. Imidazole and Derivatives. Part 1', Interscience Publishers Inc., New York, 1953, p.224.

6 D.N.Harcourt, N.Taylor, and R.D.Waigh, J.Chem.Res., 1978, (S)154, (M)1954.

7 J.N.Wells, O.R.Tarwater, and P.E.Mani, J.Org.Chem., 1972, 37, 2158.

SYNTHETIC COMMUNICATIONS, 16(7), 779-784 (1986)

A CONVENIENT PROCEDURE FOR THE REDUCTIVE DESULPHURISATION OF THIOETHERS WITH NICKEL BORIDE

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Nickel boride, conveniently prepared *in situ* from nickel (II) chloride hexahydrate and sodium borohydride, cleanly desulphurises thioethers in excellent yields.

Research into the use of alkylthio and arylthio moieties as removable activating groups in the synthesis of N-heterocycles[1,2] prompted the reappraisal of desulphurisation techniques for the reductive cleavage of thioether bonds from aromatic systems. The most widely used method to date involves a Raney nickel catalyst[3] which possesses certain drawbacks including cleavage and reduction of many other functional groups[4-7]; the highly pyrophoric nature of the catalyst and its subsequent deactivation on storage make standardisation of reactions difficult.

During our initial studies we observed that alkylthiobenzene derivatives were more efficiently desulphurised with nickel boride than with Raney nickel[8]. Nickel boride was first reported to bring about desulphurisation in the early sixties[9,10], but since then little use has been made of this reagent.

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Owing to the fact that nickel boride can be easily prepared *in situ*[9,10] from stable nickel chloride hexahydrate and sodium borohydride and the catalyst appears to be completely non-pyrophoric, a reappraisal of its utility in reductive desulphurisations appeared appropriate.

Our findings are listed in the Table and clearly demonstrate that various ethylthio, methylthio and phenylthio compounds could be cleanly desulphurised in high yields with nickel boride provided that sufficient active catalyst was formed (compare reactions 1a-c). The most cost effective molar ratio of substrate: nickel chloride hexahydrate: sodium borohydride was 1: 6: 30. In a typical experiment the sodium borohydride was added portionwise to an ice-cold methanolic solution of the substrate and nickel chloride hexahydrate, followed by stirring at room temperature overnight. The work up procedure was found to affect the yield of recovered product especially in the case of the desulphurisation of alkylthiobenzylamines (for example, compare reactions 6a and 6b) when the product was strongly adsorbed onto the catalyst. This problem was overcome by dissolution of the catalyst and product in hydrochloric acid followed by basification with ammonia to form a nickel complex before extraction of the product.

In addition to desulphurisation concomitant reduction of carbonyl groups to alcohols occurred under the reaction conditions employed, but the use of other transition metal salts or the use of isolated nickel boride may allow even greater selectivity.

Our findings illustrate that nickel boride is more selective than Raney nickel in the desulphurisation of benzaldehydes and benzylalcohols in that Raney nickel has been reported to yield toluene[4] whereas nickel boride gives good yields of benzylalcohol.

These preliminary investigations have shown that nickel boride is a convenient and effective reagent for the reductive desulphurisation of thicethers and its use in place of the more traditional Raney nickel is recommended.

Desulphurisation with sodium borohydride and nickel (II) chloride hexahydrate:-General method.

The substrate (0.002mol) and nickel (II) chloride hexahydrate (see Table) were dissolved in methanol (20ml) and sodium borohydride (see Table) added portionwise over a period of 30min. to the ice-cold mixture. Evolution of hydrogen gas and a black precipitate were observed on addition of the sodium borohydride. When the addition was complete, the ice-bath was removed and stirring continued for 16-18hr. at room temperature. Work up was achieved by methods A or B.

Method A

The reaction mixture was filtered through a bed of kieselguhr and the filter-cake washed with hot methanol/0.88 ammonia (4:1, 6 x 25ml). The filtrate was evaporated under pressure. Water (50ml) was added to the residue and the product extracted with chloroform (3 x 25ml). The combined chloroform extracts were washed with brine (1 x 25ml), dried with anhydrous magnesium sulphate and evaporated under reduced pressure.

Table: Reductive desulphurisation of thioethers with nickel boride.

Reaction	Substrate	Molar ratio Substrate:$NiCl_2.6H_2O$:$NaBH_4$		Work up	Product and yield[a]
	Benzaldehyde,				
1a	4-MeS-	1	2	20	A	Benzylalcohol (32%) + 4-Methylthiobenzylalcohol (48%)
1b	4-MeS-	1	6	60	A	Benzylalcohol (75%)
1c	4-MeS-	1	10	30	B	Benzylalcohol (94%)
2	4-EtS-	1	6	60	A	Benzylalcohol (70%)
	Benzylalcohol,				
3	4-MeS-	1	6	60	A	Benzylalcohol (77%)
4	4-PhS-	1	6	60	A	Benzylalcohol (65%)
	Benzylamine,				
5	N-$PhCH_2$-3-MeS-	1	6	60	A	N-Benzylbenzylamine (70%)
6a	N-Me-4-MeS-	1	6	60	A	N-Methylbenzylamine (38%)
6b	N-Me-4-MeS-	1	6	30	B	N-Methylbenzylamine (91%)
	1,2,3,4-Tetrahydroisoquinoline,				
7	5-MeS-4-Ph_b & 7-MeS-4-Ph^-	1	10	30	B	4-Phenyl-1,2,3,4-tetrahydroisoquinoline (89%)
8	6-MeS-4-Ph-	1	6	30	B	4-Phenyl-1,2,3,4-tetrahydroisoquinoline (79%)

9	3-(4-Ethylthio-benzoyl)-3-Me-1,2,3,4-Tetrahydro-(5H)-2-benzazepine,	1	6	30	B	3-(α-hydroxybenzyl)-3-methyl-1,2,3,4-tetrahydroisoquinoline (91%) c,d
10	8-MeS-5-Ph & 6-MeS-5-Ph e	1	6	30	B	5-Phenyl-1,2,3,4-tetrahydro-(5H)-2-benzazepine (87%) d,f

(a) All products were identified by their i.r., n.m.r., and mass spectra; crude yields.

(b) 1:1 $^{w}/_{w}$ mixture (see ref.2).

(c) Hemioxalate m.p. 176-180°C.

(d) Microanalyses C,H,N were ± 0.4% of the theoretical values.

(e) 53:8 $^{w}/_{w}$ mixture (see ref.8).

(f) Hemioxalate m.p. 217-220°C.

Method B

To the ice-cooled reaction mixture was added hydrochloric acid (32% w/w, 40ml) and stirring continued for 10min. Then 0.88 ammonia (60ml) was added slowly to basify the mixture. The mixture was filtered through a bed of kieselguhr, the filter cake washed with chloroform (3 x 25ml) and this chloroform used to extract the product from the aqueous filtrate. The combined chloroform extracts were washed with brine (1 x 25ml), dried and evaporated.

Acknowledgements

We thank the S.E.R.C. for an award (to M.R.E.).

References

1. Euerby,M.R. and Waigh,R.D., J.Chem.Research,1982, (S) 240,(M) 2417.

2. Euerby,M.R. and Waigh,R.D., J.Chem.Soc.Chem.Commun.,1984,127.

3. Pettit,G.R. and van Tamelan,E.E., Org.React.,1962,12,356.

4. Mozingo,R.,Spencer,C. and Folkers,K., J.Am.Chem.Soc., 1944,66,1859.

5. Sorkin,E.,Krahenbuhl,W. and Erlenmeyer,H., Hel.Chim.Acta, 1948,31,65.

6. Elderfield,R.C. and Clafin,E.F., J.Am.Chem.Soc.,1952,74,2953.

7. Cavalieri,L.F.,Tinker ,J.F. and Bendich,A., J.Am.Chem.Soc., 1949,71,533.

8. Euerby,M.R., PhD Thesis 1983, University of Manchester.

9. Truce,W.E. and Roberts,F.E., J.Org.Chem.,1963,28,961.

10. Truce,W.E. and Perry,F.M., J.Org.Chem.,1965,30,1316.

36 J. CHEM. RESEARCH (S), 1987

Regioselectivity in the Synthesis of Isoquinolines with Methoxy Activating Groups

J. Chem. Research (S), 1987, 36–37
J. Chem. Research (M), 1987, 0501–0526

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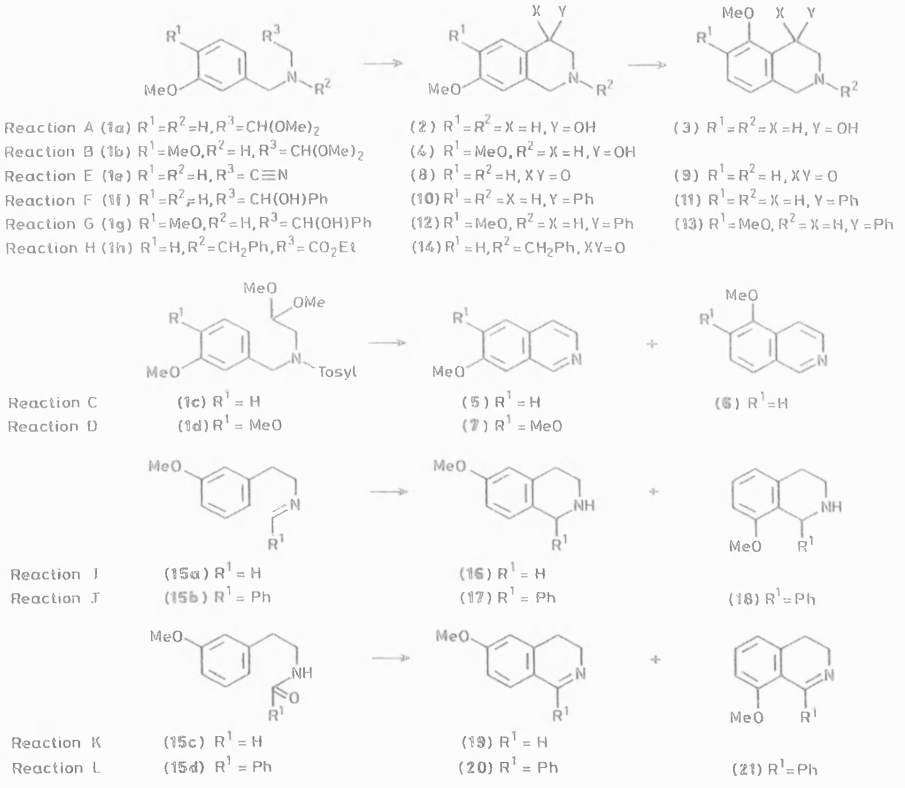
It has generally been assumed that isoquinoline syntheses involving acid-catalysed attack of a side-chain on an electron-rich benzene ring will occur preferentially *para* rather than *ortho* to a methoxy substituent, where both positions are free.[2–5] The observation that this is certainly not always true with closely analogous methylthio activating groups[6] has led us to re-examine some of the reactions previously reported and to quantify the products using crude rather than crystallised material. In most cases cyclisation involving a benzylamine intermediate and those proceeding *via* a phenylethylamine. The methods described in the literature were followed closely, up to the stage before purification, where the crude material was examined by ^{1}H n.m.r. and/or g.c., using authentic material where appropriate. The results are summarised in Table 1.

It is clear that, unlike cyclisations activated by a hydroxy group where regioselectivity is pH dependent,[12] the directing effect of a methoxy group tends to be *para* for both electronic[8] and presumably steric reasons. It is curious, however, that aminonitrile and phenylethanolamine side-chains (reactions E–G) appear to be less affected by steric considerations, and that a bulky phenyl substituent in Pictet-Spengler and Bischler-Napieralski reactions (J and L) tends to result in more product with adverse steric interactions, rather than less.



We thank the S.E.R.C. for an award (to M. R. E.) and Dr R. E. Banks for assistance with the use of hydrogen fluoride.

* To receive any correspondence.

Table 1 *Ortho* : *para* ratios and crude yields of methoxyisoquinolines

Reaction	Cyclisation agent	Isomer composition (%)[a]		*ortho* : *para* ratio
		ortho	*para*	
A	HCl	9	55	0.16
B	HCl		70	
C	HCl	4	71	0.06
D	HCl		70	
E	H_2SO_4	7	20	0.35
	HF	55	38	1.45
F	PPA[b]	45	53	0.85
	TFA[c]	37	47	0.79
G	PPA[b]	9	81	0.11
	TFA[c]	10	74	0.14
H	H_2SO_4		34	
I	HCl		58	
J	TFA[c]	2	85	0.02
K	$POCl_3$		26	
L	$POCl_3$	8	79	0.10

[a]Determined by gas chromatography and/or ^1H n.m.r. spectroscopy.
[b]Polyphosphoric acid.
[c]Trifluoroacetic acid.

Techniques used: I.r., ^1H n.m.r., mass spec., g.c.

References: 24

Tables 2–4: Spectroscopic data for the 1,2,3,4-tetrahydroisoquinolines

Tables 5–7: Spectroscopic data for the isoquinolines

Tables 8–10: Spectroscopic data for the 1,2-dihydroisoquinolin-4(3*H*)-ones

Tables 11–13: Spectroscopic data for the *N*-benzyl-1-phenyl-2-aminoethanols

Tables 14–16: Spectroscopic data for the 3,4-dihydroisoquinolines

Scheme 1: Postulated fate of *ortho* and *para* isomers in concentrated sulphuric acid

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References cited in this synopsis

2 J. M. Bobbitt, J. M. Kiely, K. L. Khanna, and R. Ebermann, *J. Org. Chem.*, 1965, **30**, 2247.
3 A. J. Birch, A. H. Jackson, and P. V. R. Shannon, *J. Chem. Soc., Perkin Trans. 1*, 1974, 2185.
4 R. Paul, J. A. Coppola, and E. Cohen, *J. Med. Chem.*, 1972, **15**, 720.
5 S. Ishiwata and K. Itakura, *Chem. Pharm. Bull.*, 1968, **16**, 778.
6 M. R. Euerby and R. D. Waigh, *J. Chem. Soc., Chem. Commun.*, 1984, 127.
8 M. R. Euerby, R. D. Waigh, I. H. Hillier, and J. P. Huke, *J. Chem. Soc., Perkin Trans. 2*, 1985, 1151.
12 H. A. Bates, K. Bagheri, and P. M. Vertino, *J. Org. Chem.*, 1986, **51**, 3061.

38 J. CHEM. RESEARCH (S), 1987

The Synthesis of Isoquinolines using Methylthio Activating Groups

J. Chem. Research (S), 1987, 38–39
J. Chem. Research (M), 1987, 0527–0553

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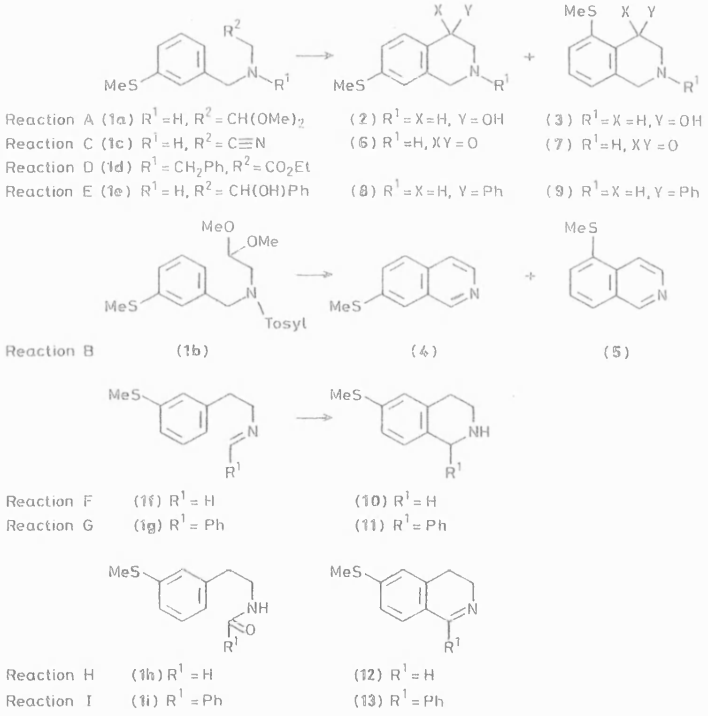
The majority of isoquinoline syntheses involve acid-catalysed ring closure to a benzene ring and benefit considerably from the presence of an electron-donating substituent.[2-3] The use of methylthio as the activating group allows the cyclisation to proceed in high yield at moderate temperatures: the methylthio group can then be removed cleanly to give the unsubstituted benzene ring.[8] In order to establish the scope of the reaction, a wide range of isoquinoline syntheses was chosen, using both benzylamine and phenylethylamine intermediates, with a variety of functional side-chains. In most cases, reaction conditions for the cyclisations which were successful for the methoxy analogues[11-18] were equally effective for the methylthio compounds. Yields were also comparable, the methylthio derivatives even giving somewhat better yields in a few instances. The yields and *ortho*:*para* ratios are given in Table 1.

A striking feature of the results is the relatively large amounts of several products formed by cyclisation *ortho* rather than *para* to the activating group. To a certain extent this can be explained using frontier orbital theory.[9] The methylthio activating groups have been successfully removed from selected tetrahydroisoquinolines by reductive desulphurisation using nickel boride; an investigation into the potential of this reagent has been reported elsewhere.[25]

We thank the S.E.R.C. for an award (to M. R. E.) and Dr. R. E. Banks for assistance with the use of hydrogen fluoride.



Techniques used: I.r., ^{1}H n.m.r., mass spec., and g.c.

References: 29

* To receive any correspondence.

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Table 1 *Ortho* : *para* ratios and crude yields of methylthioisoquinolines

Reaction	Cyclisation agent	Isomer composition (%)[a]		*ortho* : *para* ratio
		ortho	*para*	
A	HCl	37	49	0.76
B	HCl	7	47	0.15
C	H_2SO_4	7.5	5.5	1.36
	HF	69	7	9.86
D	H_2SO_4	b		
	HF	c		
E	PPA[d]	42	46	0.91
	TFA[e]	37	42	0.88
	HF	38	40	0.95
F	HCl		58	
G	TFA[e]		94	
H	$POCl_3$		21	
I	$POCl_3$		89	

[a]Determined by gas chromatography and/or ^{1}H n.m.r. spectroscopy.
[b]No chloroform extractable material recovered.
[c]Quantitative recovery of starting material.
[d]Polyphosphoric acid.
[e]Trifluoroacetic acid.

References cited in this synopsis

1 W. M. Whaley and T. R. Govindachari, *Org. React.*, 1951, **6**, (a) 74; (b) 151; (c) W. J. Gensler, *ibid.*, p. 191.
2 H. Kondo and E. Ochiai, *J. Pharm. Soc. Jpn.*, 1923, **495**, 313 (*Chem. Abstr.*, 1923, **17**, 3032).
3 J. M. Bobbitt, J. M. Kiely, K. L. Khanna, and R. Ebermann, *J. Org. Chem.*, 1965, **30**, 2247.
8 M. R. Euerby and R. D. Waigh, *J. Chem. Soc., Chem. Commun.*, 1984, 127.
9 M. R. Euerby, R. D. Waigh, I. H. Hillier, and J. P. Huke, *J. Chem. Soc. Perkin Trans. 2*, 1985, 1151.
11 G. Grethe, H. L. Lee, M. Uskokovic, and A. Brossi, *J. Org. Chem.*, 1968, **33**, 491.
12 A. J. Birch, A. H. Jackson, and P. V. R. Shannon, *J. Chem. Soc., Perkin Trans. 1*, 1974, 2185.
13 D. N. Harcourt, N. Taylor, and R. D. Waigh, *J. Chem. Soc., Perkin Trans. 1*, 1978, 722.
14 S. Ishiwata and K. Itakura, *Chem. Pharm. Bull.*, 1968, **16**, 778.
15 H. Corrodi and G. Jonsson, *Helv. Chim. Acta*, 1966, **49**, 798.
16 J. M. Bobbitt and J. C. Sih, *J. Org. Chem.*, 1968, **32**, 856.
17 R. Paul, J. A. Coppola, and E. Cohen, *J. Med. Chem.*, 1972, **15**, 720.
18 L. Helfer, *Helv. Chim. Acta*, 1924, **7**, 945.
25 M. R. Euerby and R. D. Waigh, *Syn. Commun.*, 1986, **16**, 779.

40 J. CHEM. RESEARCH (S), 1987

Methylthio Activating Groups in the Synthesis of Tetrahydroisoquinolines and Tetrahydro-2-benzazepines from *N*-Allyl- and *N*-Cinnamyl-benzylamines

J. Chem. Research (S), 1987, 40–41
J. Chem. Research (M), 1987, 0554–0575

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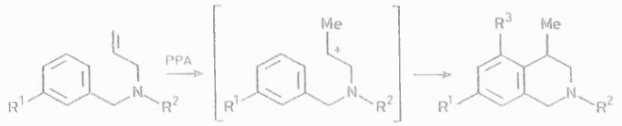
It is possible to cyclise *N*-allyl- and *N*-cinnamyl-benzylamines using polyphosphoric acid (PPA) at high temperatures to give 4-methyl-1,2,3,4-tetrahydroisoquinolines and 5-phenyl-2,3,4,5-tetrahydro-1*H*-2-benzazepines respectively. In the latter case yields are poor owing to extensive polymerisation of the starting material, but this adverse effect can be overcome by the use of a methylthio group which facilitates cyclisation and can then be reductively removed.

Previous work with *N*-allylbenzylamines had shown that the *N*,*N*-dimethyl quaternary salts could be cyclised with PPA[1] at high temperatures (300 °C). It was readily established that similar conditions (180 °C) would cyclise the secondary and tertiary bases (**1**) to give the 4-methyltetrahydroisoquinolines in high yield (see Scheme A). By analogy with the benzylaminopropynes[2] it was expected that *N*-cinnamylbenzylamines (**3**) would give the 2-benzazepines (**4**) through the phenyl-stabilised carbonium ion (Scheme B). In benzene rings to electrophilic attack in both *ortho* and *para* positions.[6] This was also effective with the *N*-cinnamyl-benzylamines (**3c**) which gave a mixture of the 6- and 8-methylthio-2-benzazepines (**4d** and **e**) in *ca.* 60% crude yield, with the 8-methylthio compound predominating. Desulphurisation of the crude mixture gave the 2-benzazepine (**4a**) in an overall yield of 53%.

Cyclisation of *N*-allyl-3-methylthiobenzylamines (**1c** and **d**) gave 4-methyltetrahydroisoquinolines (**2c–e**) as expected, at substantially lower temperatures than with the unactivated analogues (**2a**), but with the remarkable feature that cyclisation *ortho* to the methylthio group was strongly preferred to attack at the *para* position [see Scheme A, (**2d**) and (**2e**) preferred]. Production of the 5-methylthio derivative appears to be sterically very unfavourable but electronically advantageous.[10]

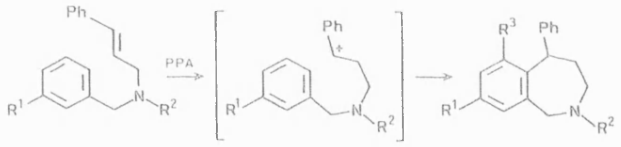
Cyclisation of the methoxyallylamine (**1e**) failed to give



(**1**) **a**; $R^1 = R^2 = H$
b; $R^1 = H$, $R^2 = Me$
c; $R^1 = MeS$, $R^2 = H$
d; $R^1 = MeS$, $R^2 = Me$
e; $R^1 = MeO$, $R^2 = H$

(**2**) **a**; $R^1 = R^2 = R^3 = H$ (91%)
b; $R^1 = R^3 = H$, $R^2 = Me$ (95%)
c; $R^1 = MeS$, $R^2 = R^3 = H$ (3%)
d; $R^1 = R^2 = H$, $R^3 = MeS$ (81%)
e; $R^1 = H$, $R^2 = Me$, $R^3 = MeS$ (84%)

Scheme A



(**3**) **a**; $R^1 = R^2 = H$
b; $R^1 = H$, $R^2 = Me$
c; $R^1 = MeS$, $R^2 = H$
d; $R^1 = MeO$, $R^2 = H$

(**4**) **a**; $R^1 = R^2 = R^3 = H$ (5%)
b; $R^1 = R^3 = H$, $R^2 = Me$ (6%)
c; $R^1 = MeS$, $R^2 = R^3 = H$ (52%)
d; $R^1 = R^2 = H$, $R^3 = MeS$ (8%)
e; $R^1 = MeO$, $R^2 = R^3 = H$ (39%)
f; $R^1 = R^2 = H$, $R^3 = MeO$ (43%)

Scheme B

practice, yields were very poor (*ca.* 5%), presumably because intermolecular reactions involving the carbonium-ion intermediate were favoured over the formation of a seven-membered ring.

We have shown that methylthio substituents will activate any isolable product on treatment with PPA, although starting material was consumed. The methoxy cinnamylamine (**3d**), however, gave a mixture of 6- and 8-methoxy-2-benzazepines (**4e** and **f**).

We thank the S.E.R.C. for an award (to M. R. E.) and Dr. B. E. Mann for assistance with nuclear Overhauser experiments.

* To receive any correspondence.

Techniques used: I.r., ^{1}H and ^{13}C n.m.r., mass spec., g.c., h.p.l.c.

References: 16

Tables 1–4: Analytical and spectroscopic data for the *N*-allyl- and *N*-cinnamyl-benzylamines (1) and (3)

Scheme 1: Polyphosphoric acid cyclisation of the *N*-allylbenzylamines (1)

Scheme 2: Polyphosphoric acid cyclisation of the *N*-cinnamylbenzylamines (3)

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References cited in this synopsis

1 S. D. Venkataramu, G. D. Macdonell, W. R. Purdum, G. A. Dilbeck, and K. D. Berlin, *J. Org. Chem.*, 1977, **42**, 2195.
2 J. R. Brooks and D. H. Harcourt, *J. Chem. Soc. C.*, 1969, 625.
6 M. R. Euerby and R. D. Waigh, *J. Chem. Soc., Chem. Commun.*, 1984, 127.
10 M. R. Euerby, R. D. Waigh, I. H. Hillier, and J. P. Huke, *J. Chem. Soc., Perkin Trans. 2*, 1985, 1151.

separated. The solvent is evaporated and the crude product is chromatographed on a silica gel column (20 cm × 3.5 cm; 70–230 mesh) using toluene (400 mL) as eluent. After evaporation of the solvent, diphenylphosphine is distilled off (100 °C/0.01 mbar) to yield a colorless oil. Phosphines **11**–**13** crystallize on trituration with MeOH (Table 3).

(*R*)-(−)-2-Benzoyloxy-3,3-dimethyl-1,4-bis(diphenylphosphino)butane (21):

A solution of phosphine **20** (12.1 g, 25.7 mmol) in toluene (60 mL) is cooled to 0 °C and benzoyl chloride (4 mL, 34.5 mmol) is added. After stirring the mixture for 10 min at room temperature, the solvent is evaporated. Trituration of the remaining yellow oil with petroleum ether (bp 40–60 °C) yields colorless crystals. The partly oxidized product is dissolved in benzene (20 mL) and reduced in an autoclave with an excess of trichlorosilane (3 mL) to pure phosphine **21**, which is again crystallized from petroleum ether.

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(1) Brunner, H. *Synthesis* **1988**, 645.
(2) Brunner, H. *Top Stereochem.* **1988**, *18*, 129.
(3) Kagan, H.B., in: *Asymmetric Synthesis*, Morrison, J.D. (ed.), Academic Press, Orlando, FL, 1985, Vol. 5, p. 1.
(4) Ito, Y., Sawamura, M., Hayashi, T. *J. Am. Chem. Soc.* **1986**, *108*, 6405.
(5) Hayashi, T., Yamamoto, A., Hagihara, T., Ito, Y. *Tetrahedron Lett.* **1986**, *27*, 191.
(6) Hayashi, T. *Pure Appl. Chem.* **1988**, *60*, 7.
(7) Vriesema, B.S., Kellogg, R.M. *Tetrahedron Lett.* **1986**, *27*, 2049.
(8) Herdeis, C. *Synthesis* **1986**, 232.
(9) Ravid, U., Silverstein, R.M., Smith, L.R. *Tetrahedron* **1978**, *34*, 1449.
(10) Nemoto, H., Nagai, M., Fukumoto, K., Kametani, T. *J. Org. Chem.* **1985**, *50*, 2764.
(11) Barbier, P., Benezra, C. *J. Med. Chem.* **1982**, *25*, 943.
(12) Fryzuk, M.D., Bosnich, B. *J. Am. Chem. Soc.* **1977**, *99*, 6262.
(13) Fryzuk, M.D., Bosnich, B. *J. Am. Chem. Soc.* **1978**, *100*, 5491.
(14) Hanessian, S., Ugolini, A., Dubé, D., Glamyan, A. *Can. J. Chem.* **1984**, *62*, 2146.
(15) Broquet, C., Bedin, J. *Bull. Soc. Chim. Fr.* **1967**, 1909.
(16) Katsura, H. *Nippon Kagaku Zasshi* **1956**, *77*, 1789; *C.A.* **1959**, *53*, 5127.
(17) Taniguchi, M., Koga, K., Yamada, S. *Tetrahedron* **1974**, *30*, 3547.
(18) Matsuo, T., Mori, K., Matsui, M. *Tetrahedron Lett.* **1976**, 1979.
(19) Amrani, Y., Lafont, D., Sinou, D. *J. Mol. Catal.* **1985**, *32*, 333.

A Modified Synthesis of 4-Chromanones

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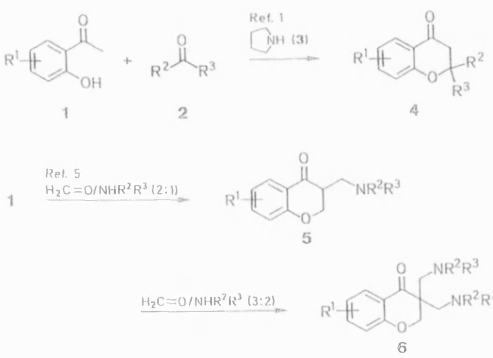
Problems in the synthesis of 4-chromanones by condensation of 2-hydroxyacetophenones with formaldehyde can be avoided by the isolation of the Mannich base hydrochlorides and cyclization by titration with potassium hydroxide.

A very simple and effective route to 4-chromanones involves the base-catalyzed condensation and cyclization of an aldehyde or ketone with 2-hydroxyacetophenone.[1] A limitation of this approach occurs with formaldehyde. The sodium salt of 2-hydroxyacetophenone in aqueous formaldehyde at 50 °C gives only a polymeric material.[2] Interestingly, the sodium salt of 2-hydroxypropiophenone under the same conditions yields 27–50% of 3-methyl-4-chromanone.[2,3] Pyrrolidine is used widely as a basic catalyst in these reactions.[1] Normally a mixture of the appropriate 2-hydroxyacetophenone **1**, aldehyde or ketone **2**, and pyrrolidine **3**, in the ratio of about 1:1:0.5 in toluene is allowed to stand for a time and then heated to boiling in an apparatus fitted with a water separator.[1]



A mixture of 2-hydroxyacetophenone, aqueous formaldehyde, and pyrrolidine in the ratio of 1:1:0.5 under these conditions yields no 4-chromanone, but a multi-component mixture of unidentifiable polar/polymeric compounds.[4] Increasing equivalents of aqueous formaldehyde and secondary amine result in the formation of 3-aminomethyl-4-chromanones **5** and 3,3-bis(aminomethyl)-4-chromanones **6**.[5]

We have been able to show that Mannich base hydrochlorides **7a–d**, formed from 2-hydroxyacetophenone by reaction with formaldehyde and dimethylamine under acidic conditions[6] will cyclize to 4-chromanones **8a–d** on titration with potassium hydroxide. If allowance is made for recovered starting material the yield on this final step is good. The 4-chromanones are obtained in pure form very readily since they are non-basic, so that separation from the starting material is very simple.

7, 8	R^1	R^2
a	H	H
b	Cl	H
c	H	Cl
d	Cl	Cl

2-Hydroxyacetophenones were prepared by the method of Jucker and Vogel[7] from the corresponding phenyl acetates. Phenyl acetates were prepared by *O*-acetylation of commercially available phenols.

Yields quoted are for purified materials. Melting points were obtained on a Kofler hot-stage apparatus, and are corrected. IR spectra were recorded on a Pye Unicam SP3-100 spectrophotometer. ^{1}H-NMR spectra were obtained using a Bruker WP 80 spectrometer. Mass spectra were recorded on a Kratos MS 30 spectrometer equipped with a DS-50 data system.

Table 1. Substituted Mannich Base Hydrochlorides 7 Prepared

Product	Yield (%)	mp (°C) (EtOH)	Molecular Formula[a] or Lit. mp (°C)	IR (KBr) ν (cm^{-1})	^{1}H-NMR (D_2O/DSS[b]) δ	MS (70 eV) m/z (%)
7a	45	154–160	156–157[8]	1650 (C=O)	6.9–8.1 (m, 4H); 3.4–3.8 (m, 4H); 2.7 (s, 6H)	193 (M^+, 1.9); 58 (100)
7b	22	195–197	188–189[9]	1645 (C=O)	6.85–7.9 (m, 3H); 3.5–3.6 (m, 4H); 2.85 (s, 6H)	229 (M^+, 0.3); 227 (M^+, 1.0); 58 (100)
7c	25	179–181	$C_{11}H_{15}Cl_2NO_2$ (264.2)	1650 (C=O)	6.9–7.8 (m, 3H); 3.45–3.55 (m, 4H); 2.85 (s, 6H)	229 (M^+, 0.6); 227 (M^+, 1.7); 58 (100)
7d	7	185	$C_{11}H_{14}Cl_3NO_2$ (298.6)	1640 (C=O)	7.95 (s, 1H); 7.05 (s, 1H); 3.5–3.6 (m, 4H); 2.85 (s, 6H)	263 (M^+, 0.4); 261 (M^+, 0.6); 58 (100)

[a] Satisfactory microanalyses obtained: C ±0.4, H ±0.2, N ±0.2.
[b] Internal standard, sodium 2,2-dimethyl-2-silapentane-5-sulfonate.

Table 2. Substituted 4-Chromanones 8 Prepared

Product	Yield (%)	Starting Material Recovered (%)	Conversion (%)	mp (°C)	Lit. mp (°C)	IR (KBr) ν (cm^{-1})	^{1}H-NMR ($CDCl_3$/TMS) δ, J (Hz)	MS (70 eV) m/z (%)
8a	50	32	74	36–37	38.5[10]	1670 (C=O)	6.9–7.95 (m, 4H); 4.45–4.6 (t, 2H, J = 6.5); 2.7–2.9 (t, 2H, J = 6.5)	148 (M^+, 67.3); 120 (100)
8b	38	52	79	101–103	106[11]	1670 (C=O)	6.9–7.9 (m, 3H); 4.5–4.7 (t, 2H, J = 6.5); 2.7–2.9 (t, 2H, J = 6.5)	184 (M^+, 18.4); 182 (M^+, 57.2); 154 (100)
8c	35	37	56	65–67	72–74[12]	1670 (C=O)	6.9–7.9 (m, 3H); 4.5–4.7 (t, 2H, J = 6.5); 2.7–2.9 (t, 2H, J = 6.5)	184 (M^+, 19.1); 182 (M^+, 59.6); 181 (100)
8d	34	34	52	126–132	131–133[13]	1670 (C=O)	7.95 (s, 1H); 7.15 (s, 1H); 4.45–4.65 (t, 2H, J = 6.5); 2.7–2.9 (t, 2H, J = 6.5)	220 (M^+, 5.9); 218 (M^+, 34.7); 216 (M^+, 56.0); 188 (100)

Mannich Base Hydrochlorides 7a–d; General Procedure:
A stirred mixture of the 2-hydroxyacetophenone (0.05 mol), paraformaldehyde (3.0 g, 0.1 mol), $Me_2HN \cdot HCl$ (6.08 g, 0.05 mol), 2-propanol (25 mL) and concentrated HCl (0.1 mL) is heated at reflux temperature for 5 h. After this time the mixture is cooled to 0°C whereupon crystallization occurs. The mixture is triturated with acetone (20 mL) and the crystalline material is filtered off. In each case the product is recrystallized from abs. EtOH. Product **7d**, although crystalline, has a deep-blue coloration which cannot be removed even with repeated recrystallization. The material is therefore dissolved in abs. EtOH (50 mL) and boiled with activated charcoal (1 g) for 10 min. The charcoal is filtered off and EtOH is evaporated off to yield a pale-yellow crystalline solid.

4-Chromanones 8a–d; General Procedure:
The appropriate Mannich base hydrochloride 7a–d (0.5 mmol) is dissolved in and made up to 25 mL with H_2O. The solution is then titrated with aq. KOH (0.02 M). During the titration the solution is maintained at 35°C between pH 3.0 and pH 11.0, and the pH is monitored with a pH-meter. The addition rate of KOH is such as to give a total time for titration of approximately 15 min. The titration is stopped when no further increase in pH is observed. After the titration the mixture is cooled to r.t. Any solid that has formed is filtered off and dried. The mixture is shaken with Et_2O (3 × 50 mL), the ethereal extracts are combined, dried ($MgSO_4$), and the solvent is evaporated. The residue is redissolved in Et_2O (20 mL) and saturated ethereal HCl is added until no further precipitation occurs. The solid material is filtered off and dried, and the ethereal filtrate is evaporated to dryness.

In each case the material precipitated by addition of ethereal HCl is found to be the starting material (Mannich bases 7a–d). The material obtained from the evaporation of the ethereal filtrate is found to be the corresponding 4-chromanone 8a–d. The titration of Mannich base 7d produces crystalline 6,7-dichloro-4-chromanone (**8d**) during the titration.

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(1) Kabbe, H.J., Widdig, A. *Angew. Chem.* **1982**, *94*, 254; *Angew. Chem. Int. Ed. Engl.* **1982**, *21*, 247, and references cited therein.
(2) Da Re, P., Verlicchi, L. *Experientia* **1960**, *16*, 301.
(3) Tilak, B.D., Muljiani, Z. *Tetrahedron* **1968**, *24*, 949.
(4) Cox, B., Waigh, R.D., unpublished observation.
(5) Cascaval, A. *Synthesis* **1983**, 579.
(6) Kuliev, A.M., Guseinov, M.S., Sardarova, S.A. *Uch. Zap. Azerb. Gos. Univ. Ser. Khim. Nauk* **1971**, *3*, 39. From Ref. *Zh. Khim.* **1972**, Abstr. No 8Zh402; *C.A.* **1973**, *79*, 91939.
(7) Jucker, E., Vogel, A. *Helv. Chim. Acta* **1963**, *46*, 727.
(8) Atwal, M.S., Bauer, L., Dixit, S.N., Gearien, J.E., Megahy, M., Morris, R., Pokorny, C. *J. Med. Chem.* **1969**, *12*, 994.
(9) McHan, J.L. *Ph. D. Thesis*, University of Mississippi, 1970.
(10) Powell, S.G. *J. Am. Chem. Soc.* **1923**, *45*, 2708.
(11) Chakravarti, D., Dutta, J. *J. Indian. Chem. Soc.* **1939**, *16*, 639.
(12) Canalini, G., Degani, I., Fochi, R., Spunta, G. *Ann. Chim. (Rome)* **1967**, *57*, 1045; *C.A.* **1968**, *69*, 2798.
(13) Irsay, R.D. *Ger. Offen* 1928027 (1969); *C.A.* **1970**, *72*, 55261.

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The Cyclisation of Benzylaminonitriles. Part 7.[8] Regiospecific Formation of Methoxy-substituted Isoquinolin-4-ones using Methylthio Activating Groups

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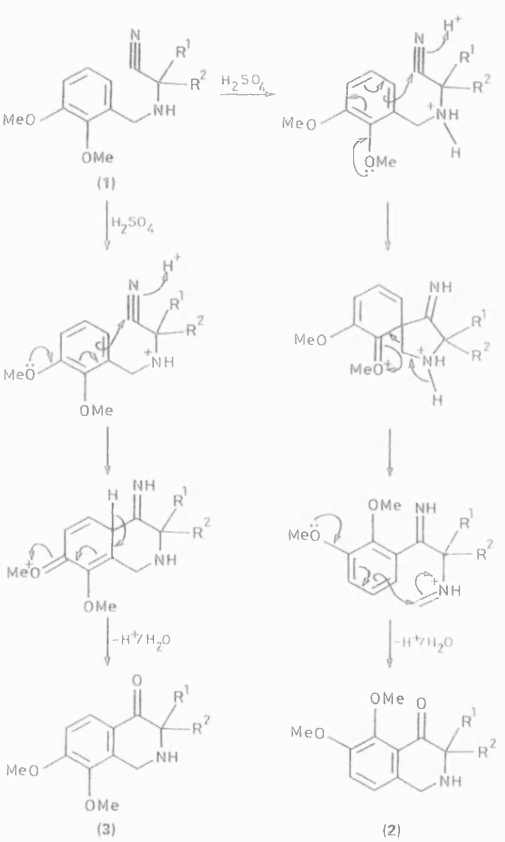
Cyclisation of 3,4-dialkoxybenzylaminoacetonitriles proceeds preferentially through a spiro intermediate. A 2,3-dialkoxy analogue will therefore normally give a 5,6-dialkoxyisoquinolinone after rearrangement and 7,8-dimethoxy substitution is not obtained. It is shown that a suitably placed methylthio group, which can be removed after cyclisation, can change the mode of cyclisation from rearrangement *via* a spiro intermediate to simple *ortho*-attack, giving the desired 7,8-dialkoxy substitution pattern. In principle, this approach to regiocontrol could be of value in many reactions involving electrophilic attack on a benzene ring.

The cyclisation of a 2,3-dimethoxybenzylaminoacetonitrile such as the cyclohexyl derivative (**1a**) has been shown to give the 5,6-dimethoxyisoquinolinone (**2a**) on treatment with sulphuric acid.[2] This can be readily explained, as in Scheme 1, by rearrangement through a spiro intermediate. In our hands this reaction also produced a small amount of 7,8-dimethoxyisoquinolinone (**3a**), presumably by orthodox cyclisation (Scheme 1); this cannot be regarded as a viable synthesis of 7,8-dimethoxyisoquinolinones, however, in view of the strong preference for the spiro route. The aminonitrile cyclisation provides an efficient route to valuable intermediates in the synthesis of benzo[*c*]phenanthridines[3,4] which makes attention to control of the substitution pattern worthwhile.

Methylthio substituents have been shown to activate benzene rings sufficiently for a variety of cyclisations involving electrophilic attack.[5–9] As an extension of the approach we envisaged several combinations of methoxy and methylthio substituents which could give the desired 7,8-dimethoxy substituted isoquinolinone, two of which are described here. To simplify isolation of the products of cyclisation we chose 2-spirocyclohexane and 2,2-dimethyl derivatives throughout, thereby avoiding problems arising through enolisation and oxidation of the isoquinolinone.

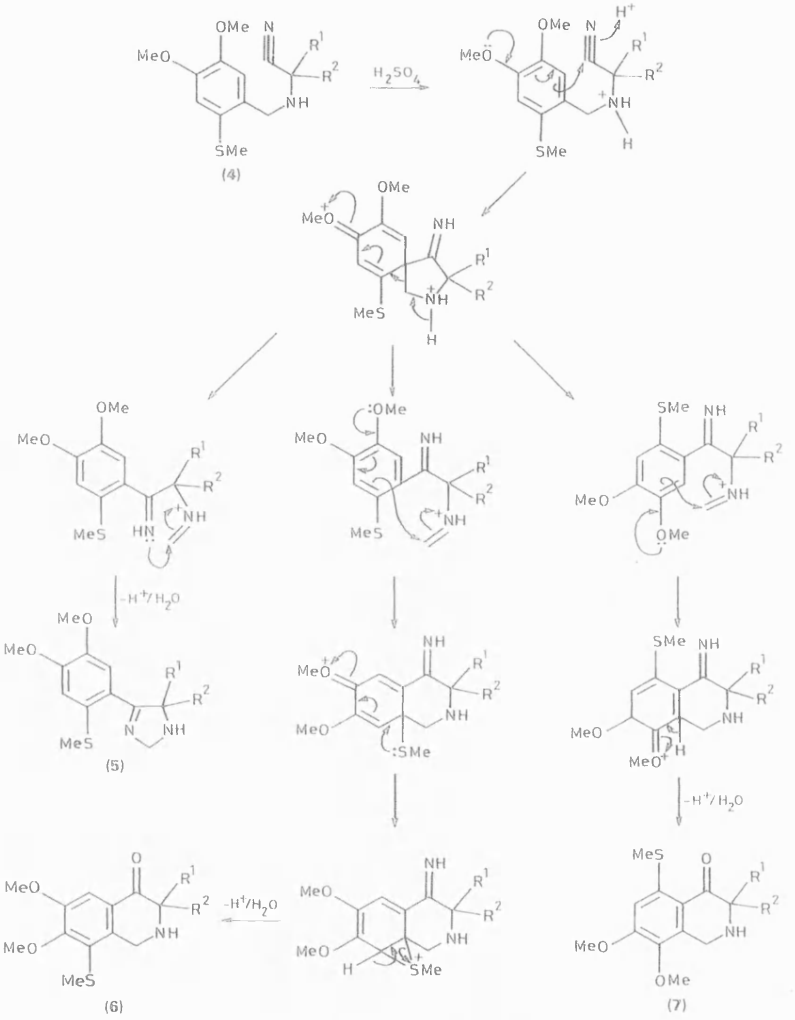


Scheme 1. a; $R^1R^2 = -(CH_2)_5-$. b; $R^1 = R^2 = Me$.

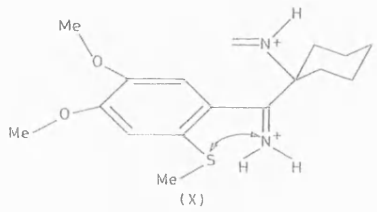
Results and Discussion

With 2-methylthio-4,5-dimethoxy substitution as in (**4a**, **b**) treatment of the aminonitrile with sulphuric acid gave fairly good yields of the imidazolines (**5a**, **b**), together with small amounts of the two isoquinolines (**6a**, **b**) and (**7a**, **b**) (Scheme 2). The imidazolines (**5a**, **b**) are characteristic of reactions in which formation of the spiro intermediate is favoured but cyclisation to the *ortho*-position of the benzene ring is inhibited. In previous examples this has been attributable to lack of a suitably placed electron donor, but this is not the case here, since the 5-methoxy group could fulfil this function and does so to a certain extent, accounting for the formation of small amounts of the desired 7,8-dimethoxyisoquinolinones (**7a**, **b**). That this secondary cyclisation does not occur readily is attributable to adverse steric interactions, as in structure (**X**). As a minor alternative, attack at the methylthio-substituted carbon followed by a 1,2 shift of MeS^+ can account for formation of the isoquinolinones (**6a**, **b**). Proof of structure of all the isoquinolinones is described below.

The 2,3-dimethoxy-5-methylthio derivatives (**8a**, **b**) had two groups activating the 6-position (Scheme 3), with the result that cyclisation proceeded by direct attack and formation of the spiro intermediates was precluded. Although yields were only moderate there were no major by-products and attention to detail is likely to bring substantial improvements; our aim of



Scheme 2. a; $R^1R^2 = -(CH_2)_5-$. b; $R^1 = R^2 = Me$.

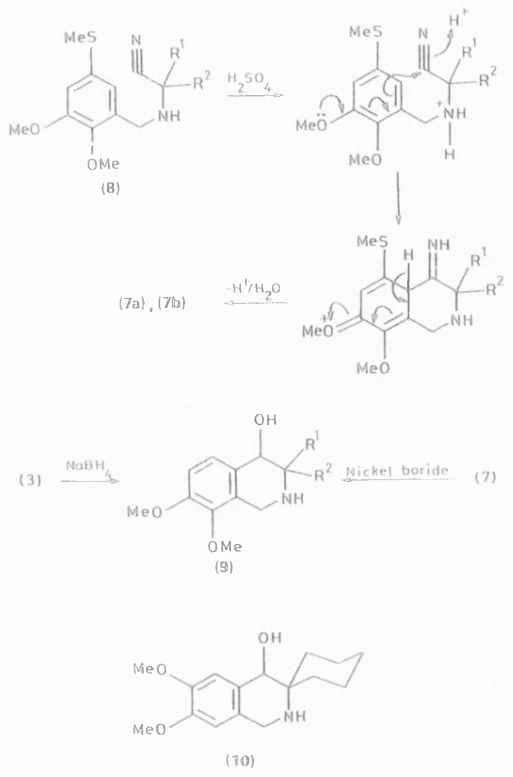


Scheme 3. a; $R^1R^2 = -(CH_2)_5-$. b; $R^1 = R^2 = Me$.

finding a simple route to 3-substituted 7,8-dimethoxy-isoquinolinones has thus been achieved.

Several methods were used to identify the cyclisation products. The isomers (2) and (3) were readily distinguished by the chemical shift differences between the two aromatic protons. In the 5,6-dimethoxy isomers (2a, b) the differences were only 0.25 and 0.24 ppm, whereas in the 7,8-dimethoxy analogues (3a, b) the differences were 0.93 and 0.92 ppm, owing to deshielding of the proton in the 5-position by the carbonyl. Reduction of these ketones with sodium borohydride gave four isomeric alcohols, two of which (9a, b) (Scheme 4) were shown to be identical with the products of nickel boride desulphurisation of (7a, b).

The identity of the 6,7-dimethoxy-8-methylthio analogue (6a)



Scheme 4. a; $R^1R^2 = -(CH_2)_5-$, b; $R^1 = R^2 = Me$.

was partly established by reductive desulphurisation to the alcohol (10). The chemical shift (δ 6.82) of the single aromatic proton in (6a) was indicative of a position *peri* to the carbonyl, and this was confirmed by examination of nuclear Overhauser effects within the molecule. Irradiation of the *S*-methyl protons in (6a) produced an enhancement of the signal from the methylene protons at position 1, and *vice versa*.

The 7,8-dimethoxy-5-methylthio analogues (7) produced in the cyclisation of (4) were shown to be identical with the products obtained from orthodox cyclisation of (8).

It is of primary importance, if this approach to regiocontrol is to be generally useful, that the appropriate synthons are readily available. A valuable method for the introduction of methylthio onto a benzene ring involves bromination and a Grignard reaction with dimethyl disulphide.[10] This approach was adopted for the synthesis of 2,3-dimethoxy-5-methylthiobenzaldehyde, starting from *o*-vanillin. The isomeric 2-methylthio-4,5-dimethoxybenzaldehyde was prepared by the method of Jacob and co-workers.[11]

Experimental

M.p.s were taken on a Reichert hot-stage apparatus and are corrected. IR spectra were obtained on a Pye-Unicam SP3-100 spectrophotometer for potassium bromide discs for solids and liquid films for oils. ^{1}H NMR spectra were recorded on Bruker WP80 and Varian SC300 instruments with $CDCl_3$ as solvent and $SiMe_4$ as internal standard. Mass spectral data were obtained with an A.E.I. MS30 instrument in the School of Chemistry, University of Manchester. Cyclisation products were separated on grade 1 basic alumina, with ethyl acetate–light petroleum (b.p. 60–80 °C) (2:1) as eluant. R_f Values were obtained using UV_{254} sensitive alumina plates, with ethyl acetate–light petroleum (b.p. 60–80 °C) (2:1) as eluant.

2,3-Dimethoxy-5-methylthiobenzaldehyde.—To *o*-vanillin (100 g, 0.66 mol) and potassium bromide (156.6 g, 1.32 mol) in 80% acetic acid (1 300 ml), bromine (35.5 ml, 0.74 mol) in glacial acetic acid (65 ml) was added, dropwise. After stirring for 15 min at 110 °C and 24 h at room temperature, water (2 500 ml) was added. The precipitate was filtered off, washed with water, and recrystallised from aqueous ethanol (50% v/v) to yield 5-bromo-2-hydroxy-3-methoxybenzaldehyde (144.3 g, 95%) as a white solid, m.p. 129 °C (lit.,[12] 128–129 °C); ν_{max} 3 200 (OH) and 1 660 (C=O) cm^{-1}; δ_H 10.94 (1 H, s, OH exchangeable), 9.86 (1 H, s, CHO), 7.31 1 H, d, *J* 1.5 Hz, ArH), 7.17 (1 H, d, *J* 1.5 Hz, ArH), and 3.92 (3 H, s, OMe); *m/z* 232 (M^+, 98%), 230 (100), 186 (37), 184 (34), 161 (13), 159 (14), 108 (22), 79 (30), 51 (69), and 29 (38).

5-Bromo-2-hydroxy-3-methoxybenzaldehyde (141 g, 0.62 mol) was dissolved in dimethylformamide (DMF; 1 200 ml), containing sodium hydroxide (24.5 g), and stirred at 70 °C for 30 min. Methyl iodide (115 ml, 1.84 mol) was added, and the mixture set aside for 12 h, then poured into water. After extraction with ether (4 × 50 ml), the solution was washed with 2M aqueous sodium hydroxide (4 × 50 ml) and water (2 × 50 ml), dried, and evaporated, and the product recrystallised from ethanol to yield 5-bromo-2,3-dimethoxybenzaldehyde (114.3 g, 76%) as a white solid, m.p. 82–83 °C (lit.,[13] 85 °C); ν_{max} 1 670 (C=O) cm^{-1}; δ_H 10.34 (1 H, s, CHO), 7.53 (1 H, d, *J* 2.5 Hz, ArH), 7.23 (1 H, d, *J* 2.5 Hz, ArH), 3.98 (3 H, s, OMe), and 3.91 (3 H, s, OMe); *m/z* 246 (M^+, 98%), 244 (M^+, 98%), 231 (30), 229 (38), 200 (30), 198 (22), 188 (48), 186 (27), 107 (33), 94 (92), 44 (100), and 29 (78).

5-Bromo-2,3-dimethoxybenzaldehyde (114.3 g, 0.47 mol) was dissolved in absolute ethanol (82 ml, 1.43 mol). Triethyl orthoformate (85.3 ml, 0.51 mol) was then added, followed by 32% hydrochloric acid (0.2 ml), whereupon the temperature rose quickly. The reaction mixture was heated under reflux on a steam bath for 30 min, then rapidly cooled, and basified with aqueous sodium hydroxide (20% w/v). The combined ethereal extracts were washed with water (50 ml), dried, and evaporated to yield 5-bromo-2,3-dimethoxybenzaldehyde diethyl acetal (133 g, 93%) as an oil, ν_{max} 1 050 (COC) cm^{-1}; δ_H 7.31 (1 H, d, *J* 1 Hz, ArH), 7.00 (1 H, d, *J* 1 Hz, ArH), 5.72 (1 H, s, CH), 3.85 (3 H, s, OMe), 3.83 (3 H, s, OMe), 3.66 (4 H, q, *J* 7 Hz, 2 × CH_2O), and 1.23 (6 H, t, *J* 7 Hz, 2 × CH_2CH_3); *m/z* 320 (M^+, 15%), 318 (M^+, 13%), 275 (100), 273 (96), 245 (37), 138 (54), 75 (42), 44 (52), and 29 (57).

5-Bromo-2,3-dimethoxybenzaldehyde diethyl acetal (133 g, 0.42 mol) was added to dry tetrahydrofuran (THF; 1 l) containing magnesium (10.25 g, 0.42 mol), under an atmosphere of nitrogen. Upon formation of the Grignard complex, dimethyl disulphide (37.7 ml, 0.42 mol), in dry THF (250 ml) was added dropwise over 30 min. The solution was heated under reflux for 3½ h and cooled, and aqueous NH_4Cl (20% w/v; 2 l) added cautiously, keeping the temperature below 20 °C, and employing a thiol trap.[10] The mixture was filtered and extracted with ether (3 × 250 ml), and the extracts were dried and evaporated to yield 2,3-dimethoxy-5-methylthiobenzaldehyde diethyl acetal (108.6 g, 91%) as an oil; ν_{max} 1 050 (COC) cm^{-1}; δ_H 7.09 (1 H, d, *J* 1 Hz, ArH), 6.82 (1 H, d, *J* 1 Hz, ArH), 5.72 (1 H, s, CH), 3.85 (3 H, s, OMe), 3.83 (3 H, s, OMe), 3.60 (4 H, q, *J* 7 Hz, 2 × CH_2O), 2.48 (3 H, s, OMe), and 1.23 (6 H, t, *J* 7 Hz, 2 × CH_2CH_3); *m/z* 286 (M^+, 47%), 241 (80), 212 (17), 195

Table 1. Spectroscopic data for benzylaminoacetonitriles.

Compound	ν_{max}/cm^{-1} NH	C≡N	*m/z*	δ_H
(1b)	3 300	2 240	234 (M^+, 0.3%), 207 (15), 192 (7.5), 176 (38) 167 (54), 165 (34), 152 (59), 151 (60), 136 (100), 121 (28), 106 (32), 77 (31)	6.97–6.91 (3 H, m, ArH), 3.89 (5 H, s, OMe and CH_2N), 3.87 (2 H, s, OMe), 1.92 (1 H, s, NH[a]), 1.52 (6 H, s, CH_3)
(4a)	3 310	2 200	293 (M^+ − 27, 15%), 278 (7), 246 (56), 197 (100), 182 (23), 151 (19), 138 (16), 27 (7)	6.92 (1 H, s, ArH), 4.0 (2 H, s, $ArCH_2N$), 3.88 (6 H, s, OMe), 2.45 (3 H, s, SMe), 1.69 (1 H, s, NH[a]), 1.83–1.54 (10 H, br, C_5H_{10})
(4b)	3 300	2 200	253 (M^+, 74%), 238 (29), 206 (64), 197 (100), 182 (53), 151 (40), 138 (40), 70 (15), 45 (10)	6.93 (1 H, s, ArH), 6.90 (1 H, s, ArH), 3.98 (2 H, s, $ArCH_2N$), 3.89 (6 H, s, OMe) 2.45 (3 H, s, SMe), 1.60 (1 H, s, NH[a]), 1.55 (6 H, s, CH_3)
(8a)	3 300	2 200	320 (M^+, 0.5%), 293 (12), 262 (36) 197 (17), 182 (48), 98 (45), 56 (88) 41 (68), 27 (100)	6.86 (1 H, s, ArH), 6.84 (1 H, s, ArH), 3.86 (8 H, s, 2 × OMe and CH_2N), 2.48 (3 H, s, SMe), 1.77 (1 H, br, NH[a]), 2.12–1.48 (10 H, br, C_5H_{10})
(8b)	3 300	2 200	253 (M^+ − 27, 47%), 238 (20), 222 (35) 213 (37), 198 (20), 197 (100), 193 (29) 161 (36), 137 (45), 77 (33), 45 (46), 30 (43)	6.83 (2 H, br, 2 ArH), 3.86 (8 H, s, 2 × OMe and $ArCH_2N$), 2.48 (3 H, s, SMe), 1.84 (1 H, br, NH[a]), 1.52 (6 H, s, 2 × CH_3)

[a] 1 H exchangeable.

Table 2. Benzylaminoacetonitriles.

Compound	Yield (%)	M.p., *t*/°C	Found (%) C	H	N	Required (%) C	H	N
(1b)	80	98–101	57.2	7.2	9.8	57.6	7.2	10.3
(4a)	78	81	63.6	7.6	8.8	63.7	7.6	8.7
(4b)	88	77	60.0	7.3	9.8	60.0	7.2	10.0
(8a)	92	119–121[a]	57.3	7.1	7.8	57.2	7.1	7.9
(8b)	88	Oil			[b]			

[a] Of hydrochloride. [b] Found: M^+, 280.1251; $C_{14}H_{20}N_2O_2S$ requires *M*, 280.1246.

(29), 138 (56), 105 (100), 77 (51), 47 (48), and 29 (15) (Found: M^+, 286.1249. $C_{14}H_{22}O_4S$ requires *M*, 286.1248).

2,3-Dimethoxy-5-methylthiobenzaldehyde diethyl acetal (108.5 g, 0.38 mol) was heated under reflux with 2M sulphuric acid (1 l) for 1 h. The mixture was rapidly cooled and extracted with ether (3 × 150 ml). The combined extracts were washed with dilute aqueous sodium hydroxide (50 ml) and water (50 ml), dried, and evaporated to yield a brown oil (49.8 g, 79%). Vacuum distillation afforded the *aldehyde* as a pale yellow oil, b.p. 117 °C at 0.05 mmHg, which rapidly solidified. Recrystallisation from ethanol yielded pale yellow needles, m.p. 60–61 °C (Found: C, 56.3; H, 5.8%. $C_{10}H_{12}O_3S$ requires C, 56.5; H, 5.7%); ν_{max} 1 680 (C=O) cm^{-1}; δ_H 10.38 (1 H, s, CHO), 7.26 (1 H, d, *J* 1.5 Hz, ArH), 7.15 (1 H, d, *J* 1.5 Hz, ArH), 3.96 (3 H, s, OMe), 3.90 (3 H, s, OMe), and 2.50 (3 H, s, SMe); *m/z* 212 (M^+, 100%), 197 (70), 169 (22), 154 (27), 141 (30), 111 (24), and 32 (15).

2-Methylthio-4,5-dimethoxybenzaldehyde.—This compound was prepared according to the method of Jacob and co-workers,[11] in 50% overall yield, m.p. 111 °C (lit.,[11] 112–113 °C).

Preparation of Benzylamines and Benzylaminoacetonitriles.—These compounds were obtained by established methods.[5]

2,3-Dimethoxy-5-methylthiobenzylamine hydrochloride was recrystallised from ethanol (95% yield over 2 steps), m.p. 153 °C (Found: C, 48.0; H, 6.5; N, 5.3%. $C_{10}H_{16}ClNO_2S$ requires C, 48.1; H, 6.5; N, 5.6%); ν_{max} 3 400 (NH) cm^{-1}; δ_H 7.21 (1 H, d, *J* 2.5 Hz, ArH), 6.78 (1 H, d, *J* 2.5 Hz, ArH), 4.24 (2 H, s, $ArCH_2N$), 4.00 (3 H, s, OMe), 3.82 (3 H, s, OMe), 2.49 (3 H, s, Me), and 2.38 (2 H, br, NH_2, exchangeable); *m/z* 214 (M^+, 11%), 213 (88), 198 (15), 182 (21), 166 (14), 151 (15), 36 (93), and 31 (100).

4,5-Dimethoxy-2-methylthiobenzylamine hydrochloride was recrystallised from ethanol (89% yield over 2 steps), m.p. 180 °C (Found: C, 48.3; H, 6.7; N, 5.4%. $C_{10}H_{16}ClNO_2S$ requires C, 48.1; H, 6.5; N, 5.6%); ν_{max} 3 380 (NH) cm^{-1}; δ_H 6.92 (2 H, s, ArH), 3.92 (2 H, s, CH_2N), 3.89 (6 H, s, 2 × OMe), 2.44 (3 H, s, SMe), and 1.64 (2 H, br, NH_2, exchangeable); *m/z* 213 (M^+, 76%), 212 (91), 198 (100), 181 (60), 164 (25), 151 (29), 138 (49), and 30 (36).

Cyclisation of Benzylaminoacetonitriles: General Methods.—The benzylaminoacetonitrile (2 g) in chloroform (8 ml) was added to 94% sulphuric acid (10 ml) at 0 °C during 5 min. The mixture was then stirred for 15 min at room temperature. Work-up was then achieved using method A or B.

Method A. The solution was added dropwise to ice–water and stirred for 5 min after which it was added slowly to an ice-cooled saturated solution of sodium hydrogen carbonate. The free base was extracted with chloroform, and the extracts were washed with water, dried ($MgSO_4$), and evaporated.

Method B. The solution was added dropwise cautiously to ice–dilute aqueous ammonia. The cyclised products were extracted with chloroform, and the extracts were washed with water, dried, and evaporated (water bath < 30 °C).

Spectral and analytical data for the novel isoquinolin-4(3*H*)-one products are presented in Tables 3 and 4.

Cyclisation of 1-(2,3-dimethoxybenzylamino)cyclohexanecarbonitrile (1a). Work-up using method A yielded a brown oil (0.5 g). ^{1}H NMR and TLC of the crude mixture suggested the presence of 1,2-dihydro-5,6-dimethoxyisoquinoline-3-spiro-cyclohexan-4(3*H*)-one (2a) (R_f 0.61) and the corresponding 7,8-dimethoxy compound (3a) (R_f 0.73), in a 4:1 ratio. Partial purification of the mixture was achieved by formation of the hydrochloride salt, by addition of ethereal hydrogen chloride. Preparative TLC of the rebasified mixture gave (2a) (0.26 g, 13%), and (3a) (0.06 g, 3%) as green translucent plates. Spectroscopic properties of (2a) were identical with those found previously.[2]

Table 3. Spectroscopic data for 1,2-dihydroisoquinolin-4(3*H*)-ones.

Compound	ν_{max}/cm^{-1} NH	C=O	*m/z*	δ_H
(2b)	3 300	1 680	235 (M^+, 18%), 207 (10), 176 (27), 167 (41), 151 (36), 136 (100), 91 (46), 77 (17), 58 (10)	7.09 (1 H, d, *J* 8.4 Hz, ArH), 6.85 (1 H, d, *J* 8.4 Hz, ArH), 4.07 (2 H, s, $ArCH_2N$), 3.89 (2 H, s, OMe), 3.87 (3 H, s, OMe), 2.08 (1 H, br, NH[a]), 1.35 (6 H, s, CH_3)
(3a)	3 300	1 660	275 (M^+, 23%), 216 (52), 204 (13), 178 (11), 151 (17), 136 (17), 91 (16), 40 (100), 29 (63)	7.82 (1 H, d, *J* 8 Hz, ArH), 6.89 (1 H, d, *J* 8 Hz, ArH) 4.12 (2 H, s, NCH_2N), 3.91 (3 H, s, OMe), 3.83 (3 H, s, OMe), 1.93 (1 H, s, NH[a]), 2.12–1.35 (10 H, br, C_5H_{10})
(3b)	3 300	1 680	235 (M^+, 16%), 219 (4), 207 (10), 178 (59), 151 (100), 136 (49), 120 (11), 91 (37), 77 (14), 58 (26), 40 (47), 32 (64)	7.77 (1 H, d, *J* 4.7 Hz, ArH), 6.85 (1 H, d, *J* 4.7 Hz, ArH), 3.87 (3 H, s, OMe), 3.85 (3 H, s, OMe), 3.68 (2 H, s, $ArCH_2N$), 1.69 (1 H, br, NH[a]), 1.41 (6 H, s, CH_3)
(6a)	3 400	2 680	321 (M^+, 60%), 306 (17), 250 (48), 246 (88), 224 (31), 197 (96), 182 (58), 84 (55), 49 (84), 36 (100)	7.58 (1 H, s, ArH), 4.21 (2 H, s, $ArCH_2N$), 3.96 3.90 (3 H, s, OMe), 2.39 (3 H, s, SMe), 1.88 (1 H, s, NH[a]), 1.67–1.50 (10 H, br, C_5H_{10})
(6b)	3 320	1 660	281 (M^+, 49%), 224 (100), 208 (34), 197 (8), 182 (61), 58 (81), 29 (15)	7.59 (1 H, s, ArH), 4.28 (2 H, $ArCH_2N$), 3.97 (3 H, s, OMe), 3.94 (3 H, OMe), 2.39 (3 H, s, SMe), 2.08 (1 H, s, NH[a]), 1.36 (6 H, s, CH_3)
(7a)	3 300	1 650	321 (M^+, 60%), 306 (17), 250 (48), 246 (88), 224 (31), 197 (96), 182 (58), 84 (55), 49 (84), 36 (100)	6.68 (1 H, s, ArH), 4.12 (2 H, s, $ArCH_2N$), 3.95 (3 H, s, OMe), 3.80 (3 H, s, OMe), 2.42 (3 H, s, SMe) 2.05 (1 H, s, NH[a]), 1.84–1.68 (10 H, br, C_5H_{10})
(7b)	3 280	1 650	321 (M^+, 37%), 224 (100), 209 (41), 196 (45), 182 (50), 58 (97)	6.65 1 H, s, ArH), 4.15 (2 H, s, $ArCH_2N$), 3.92 (3 H, s, OMe), 3.78 (3 H, s, OMe), 2.42 (3 H, s, SMe), 2.10 (1 H, s, NH[a]), 1.42 (6 H, s, CH_3)

[a] 1 H exchangeable.

Table 4. 1,2-Dihydroisoquinolin-4(3*H*)-ones.

Compound	M.p., *t*/°C[a]	Found (%) C	H	N	Required (%) C	H	N
(2a)	98[b]						
(2b)	198[c]	57.6	6.8	5.2	57.4	6.7	5.2
(3a)	75	69.4	7.6	5.2	69.6	8.0	5.1
(3b)	—			*d*			
(6a)	67–68	63.8	7.2	4.3	63.5	7.2	4.3
(6b)	Oil						
(7a)	102–104	63.8	7.5	4.3	63.5	7.2	4.3
(7b)	110–112	60.1	6.8	4.9	59.8	6.8	5.0

[a] From light petroleum (b.p. 60–80 °C). [b] Lit.,[2] 106 °C. [c] Of hydrochloride (from methanol–ether, 1:1). [d] Found: M^+, 235.1179; $C_{13}H_{17}NO_3$ requires *M*, 235.1176.

Cyclisation of 2-(2,3-*dimethoxybenzylamino*)-2-*methylpropionitrile* (1b). Work-up using method A yielded a brown oil (0.66 g). ^{1}H NMR and TLC of the product suggested the presence of 1,2-dihydro-5,6-dimethoxy-3,3-dimethylisoquinolin-4(3*H*)-one (2b) (R_f 0.39), and 1,2-dihydro-7,8-dimethoxy-3,3-dimethylisoquinolin-4(3*H*)-one (3b) (R_f 0.43), in a 4:1 ratio. Formation of the hydrochloride salt followed by preparative TLC gave (2b) as yellow–green needles (0.28 g, 14%), and (3b) as a pale yellow oil (0.06 g, 3%).

Cyclisation of 1-(4,5-*dimethoxy*-2-*methylthiobenzylamino*)-*cyclohexanecarbonitrile* (4a). Work-up using method B yielded a pale oil (1.55 g). TLC showed the presence of one major product plus three minor ones. These were separated by column chromatography. The three minor components were 1,2-dihydro-6,7-dimethoxy-8-methylthioisoquinoline-3-spirocyclohexan-4(3*H*)-one (6a) (0.12 g, 6%; R_f 0.87), the starting aminoacetonitrile (4a) (0.06 g, 3%; R_f 0.75), and the corresponding 7,8-dimethoxy compound (7a) (0.04 g, 2%; R_f 0.58). The major product obtained as a colourless oil was 4-(4,5-dimethoxy-2-methylthiophenyl)-4,5-dihydroimidazole-5-spirocyclohexane (5a) (1.34 g, 67%; R_f 0.39) (Found: M^+, 320.1552; $C_{17}H_{24}N_2O_2S$ requires *M*, 320.1545); ν_{max} 3 260 (NH) and 1 590 (C=N) cm^{-1}; *m/z* 320 (M^+ 1.3%), 305 (2), 276 (3), 111 (100), 110 (23), 83 (40), and 82 (10); δ_H 6.97 (1 H, s, ArH), 6.66 (1 H, s, ArH), 4.89 (2 H, s, $ArCH_2N$), 3.92 (3 H, s, OMe), 3.86 (3 H, s, OMe), 2.40 (3 H, s, SMe), 1.85 (1 H, br, NH, exchangeable), and 1.84–1.48 (10 H, br, C_5H_{10}).

Cyclisation of 2-(4,5-*dimethoxy*-2-*methylthiobenzylamino*)-2-*methylpropionitrile* (4b). Work-up using method B yielded a pale oil (1.8 g). TLC showed one major product plus two minor ones. These were separated by column chromatography. The two minor products were identified as 1,2-dihydro-6,7-dimethoxy-3,3-dimethyl-8-methylthioisoquinolin-4(3*H*)-one (6b) (0.08 g, 4%; R_f 0.78), and 1,2-dihydro-7,8-dimethoxy-3,3-dimethyl-5-methylthioisoquinolin-4(3*H*)-one (7b) (0.06 g, 3%; R_f 0.59). The major product, obtained as a white solid was 2,5-*dihydro*-4-(4,5-*dimethoxy*-2-*methylthiophenyl*)-5,5-*dimethylimidazole* (5b) (1.2 g, 60%; R_f 0.45), m.p. 64–66 °C (Found: C, 60.1; H, 7.5; N, 9.8%. $C_{14}H_{20}N_2O_2S$ requires C, 60.0; H, 7.2; N, 10.0%); ν_{max} 3 260 (NH) and 1 590 (C=N) cm^{-1}; *m/z* 280 (M^+, 0.8%), 265 (1.4), 84 (13), 72 (36), and 71 (100); δ_H 6.96 (1 H, s, ArH), 6.74 (1 H, s, ArH), 4.89 (2 H, s, $ArCH_2N$), 3.93 (3 H, s, OMe), 2.42 (3 H, s, SMe), 2.39 (1 H, s, NH, exchangeable), and 1.36 (6 H, s, CH_3).

Cyclisation of 1-(2,3-*dimethoxy*-5-*methylthiobenzylamino*)-*cyclohexanecarbonitrile* (8a). Work-up using method A yielded a brown oil (1.05 g). TLC showed one major and one minor component which were separated by column chromatography. The major product was the dimethoxy compound (7a) (0.68 g, 34%; R_f 0.60). The minor component was uncyclised starting material (0.32 g, 16%; R_f 0.79).

Cyclisation of 1-(2,3-*dimethoxy*-5-*methylthiobenzylamino*)-2-

Table 5. 1,2,3,4-Tetrahydroisoquinolin-4-ols.

Compound	Yield (%)	ν_{max}/cm^{-1} (NH)	*m/z*	δ_H
(9a)	60[a]/48[b]	3 440	227 (M^+, 16%), 275 (21), 257 (21), 206 (19), 180 (43), 165 (24), 136 (21), 98 (100), 40 (76), 29 (51)	6.95 (2 H, ABq, *J* 7 Hz, 2 ArH), 4.14 (1 H, s, C*H*OH) 3.96 (2 H, d, *J* 3 Hz $ArCH_2N$), 3.85 (3 H, s, OMe) 3.81 (3 H, s, OMe), 2.23 br (2 H, br, NH[c] and OH[c]), 1.60–1.26 (10 H, br, C_5H_{10})
(9)	55[a]/41[b]	3 300	237 (M^+, 10%), 220 (5), 204 (3), 101 (31), 165 (11), 58 (100), 42 (53), 39 (20), 27 (23)	6.92 (2 H, ABq, *J* 8 Hz, 2 ArH), 4.19 (1 H, s, C*H*OH) 4.11 (2 H, d, *J* 4.5 Hz, $ArCH_2N$), 3.84 (3 H, s. OMe) 3.80 (3 H, s, OMe), 1.42 (2 H, br, NH[c] and OH[c]), 1.40 (3 H, s, Me), 1.29 (3 H, s, Me)
(10)	55[a]	3 340	277 (M^+, 11%), 275 (10), 257 (44), 180 (73), 166 (50), 151 (6.8), 136 (54), 98 (100), 40 (60), 29 (42)	6.82 (1 H, s, ArH), 6.40 (1 H, s, ArH), 4.10 (1 H, s, C*H*OH), 3.86 (3 H, s, OMe), 3.83 (3 H, s, OMe), 3.79 (2 H, s. $ArCH_2N$), 2.45 (2 H, br, NH[c] and OH[c]), 1.59–1.19 (10 H, br, C_5H_{10})

[a] From sodium borohydride reduction of 1,2-dihydroisoquinolin-4(3*H*)-ones. [b] From nickel boride desulphurisation of 1,2-dihydroisoquinolin-4(3*H*)-ones. [c] 1 H exchangeable.

methylpropionitrile (**8b**). Work-up using method A yielded a brown oil (0.8 g). TLC showed two products, which were identified by column chromatography as the isoquinolinone (**7b**) (0.6 g, 30%; R_f 0.47) and uncyclised starting material (0.2 g, 10%; R_f 0.84).

Sodium Borohydride Reductions.—Isoquinolin-4(3*H*)-ones (**3a**), (**3b**) and (**6a**) were reduced under standard conditions to give the corresponding 1,2,3,4-tetrahydroisoquinolin-4-ols (**9a**), (**9b**), and (**10**). Spectroscropic and analytical data are presented in Table 5.

Nickel Boride Desulphurisations.—Isoquinolin-4(3*H*)-ones (**7a**) and (**7b**) were reductively desulphurised in accordance with literature methods.[7,8] The respective products (**9a**) and (**9b**) were isolated upon work-up as uncrystallisable glasses. Spectroscopic and analytical data are presented in Table 5.

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References

1 Part 6, D. N. Harcourt, F. Hussain, N. Taylor, and M. Nasir, *J. Chem. Soc., Perkin Trans. 1*, 1986, 1329.
2 D. N. Harcourt, N. Taylor, and R. D. Waigh, *J. Chem. Soc., Perkin Trans 1*, 722, 1978.
3 T. Olugbade and R. D. Waigh, *J. Pharm. Pharmacol.*, 1981, 81P.
4 T. Olugbade, Ph.D. Thesis, Manchester University, 1983.
5 M. R. Euerby and R. D. Waigh, *J. Chem. Res.*, 1982, (*S*) 240; (*M*) 2417.
6 M. R. Euerby and R. D. Waigh, *J. Chem. Soc., Chem. Commun.*, 1984, 127.
7 M. R. Euerby, A. C. Oldroyd, and R. D. Waigh, *Heterocycles*, 1986, **24**, 2871.
8 M. R. Euerby and R. D. Waigh, *Synth. Commun.*, 1986, **16**, 779.
9 M. R. Euerby and R. D. Waigh, *J. Chem. Res.* (*S*), 1987, 38.
10 M. R. Euerby and R. D. Waigh, *Synth. Commun.*, 1981, **11**, 849.
11 P. Jacob III, G. Anderson III, C. K. Meshul, A. T. Shugin, and N. Castagnoli, Jnr, *J. Med. Chem.*, 1977, **20**, 1235.
12 M. Brink, *Acta. Univ. Lund. II*, 1967, **36**, 9.
13 A. J. Birch, D. N. Butler, R. Effenburger, and T. J. Simpson, *J. Chem. Soc., Perkin Trans. 1*, 1979, **3**, 807.

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A Very Short Route to Fully Aromatic 2,3,8,9- and 2,3,8,9,12-Oxygenated Benzo[*c*]phenanthridines

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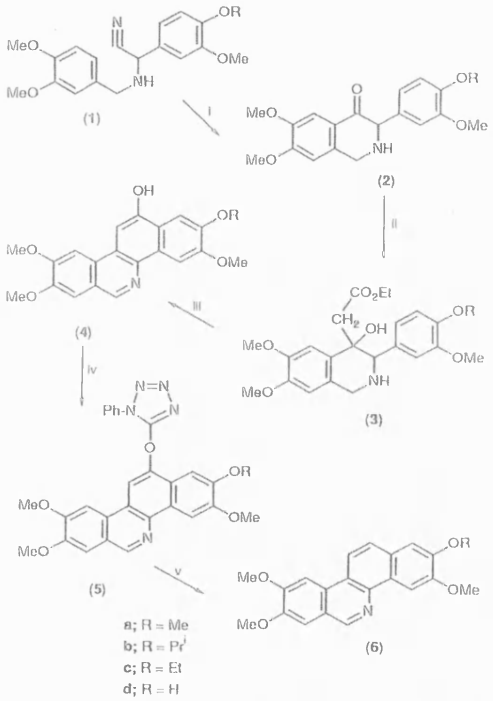
Cyclisation of suitably substituted 2-benzylamino-2-phenylacetonitriles proceeds by rearrangement, in sulphuric acid or anhydrous hydrogen fluoride, to give 3-aryl-1,2-dihydroisoquinolinones possessing all but two carbons of the benzo[*c*]phenanthridine ring system. These two carbon atoms are introduced in high yield by means of a modified Reformatski reaction and the resulting ester is cyclised in sulphuric acid, with concomitant dehydration and oxidation, to give the fully aromatic four-ring system in only four steps.

Naturally occurring 2,3,8,9-oxygenated benzo[*c*]phenanthridines are of interest for the potent antileukaemic activity (in animals) of their *N*-methyl salts.[1] The published synthetic methods are numerous, but almost without exception are very long, or include one or more photochemical steps which are not amenable to large scale reactions. The number of analogues which have been prepared for structure-action studies is therefore quite small. In contrast to the literature procedures, a two-stage synthesis of a 3-arylisoquinoline provides all but two carbons of the ring system,[2] with suitable functionalisation for the remaining ring to be added. The synthesis has now been completed (Scheme 1).

Attention to detail improved the yield of the isoquinolinone (2a) from 24% to 54%, in sulphuric acid, and was further raised to 66% in anhydrous hydrogen fluoride (HF). With a view to the synthesis of analogues of the highly potent antileukaemic alkaloid fagaronine,[3] the isopropyl derivative (1b) was prepared and cyclised, giving the phenolic isoquinolinone (2d) in both sulphuric acid and HF. Since deprotection was not desired at this early stage, the ethyl analogue (1c) was used, giving the *O*-ethyl isoquinolinone (2c). The use of HF as cyclising agent resulted in a 68% yield, compared to 29% in sulphuric acid.

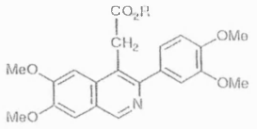
A two-carbon synthon was required, with appropriate substitution for generation of a nucleophile, and with functionality on the other carbon suitable for a subsequent acid-catalysed ring closure. The first choice was an α-halogeno acetal, but these are reported not to form stable Grignard complexes.[4] A Reformatski reaction was an alternative, although giving a different oxidation state. The normal conditions for the Reformatski reaction involve introduction of the halogeno ester and ketone into the reaction mixture together, to minimise self-condensation of the ester and zinc complex. This was not an attractive proposition when using an amino ketone which could react nucleophilically with the halogeno ester, but a modification described by Cure and Gaudemar[5] offered a way round the problem. In their method, the complex between zinc and ethyl bromoacetate is formed in the presence of dimethoxymethane. Addition of the ketone can be delayed until the complex is fully formed. At first we found that very pure zinc had to be used but the use of trimethylsilyl chloride as an initiator[6] avoids even this drawback. The method is a significant improvement and deserves to be more widely known. Using this method the esters (3a) and (3c) were obtained in yields in excess of 80%. In each case only one of the diastereoisomeric pairs was formed. We presume that attack occurs from the opposite side of the ring from the phenyl substituent, giving the isomers in which the ester and phenyl groups are *trans*. Since the next step involved dehydration the stereochemistry is not significant for the present.



Scheme 1. *Reagents and conditions*: i, H^+; ii, Reformatski; iii, H_2SO_4, iv, 5-chloro-1-phenyl-1*H*-tetrazole, K_2CO_3; v, H_2/Pd.

Cyclisation of the β-hydroxy esters (3a) and (3c) in sulphuric acid gave the fully aromatic benzo[*c*]phenanthridines (4a) and (4c) in 52 and 70% yields respectively. There was some de-ethylation in the latter reaction, giving the diphenol (4d) in up to 14% yield with longer reaction times. The conversion from β-hydroxy ester into fully aromatic four-ring system must involve

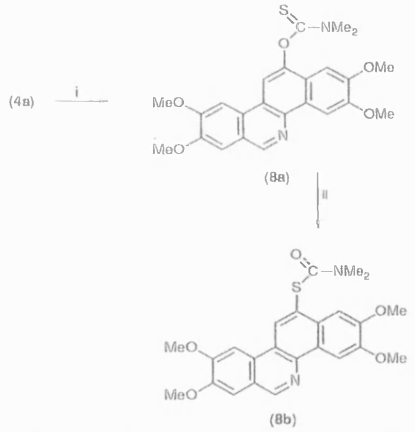
four steps, the preferred order of which is not entirely clear. Cyclisation to give a ketone and dehydration of the tertiary alcohol could occur in either order. Enolisation of the ketone would then be followed by oxidation of the 5,6-dihydrobenzo[*c*]phenanthridine by air or by sulphuric acid; such compounds are known to oxidise readily.[7] The oxidative stage is probably final, since dehydration and spontaneous aromatisation in ethanolic hydrogen chloride gave the ester (7a)



(7) a; R = Et
b; R = H

which did not cyclise in sulphuric acid. However, when the dihydrobenzo[*c*]phenanthridine was first hydrolysed using hydrochloric acid, to produce the acid (7b), the cyclisation in sulphuric acid proceeded readily. The latter procedure offers an alternative (longer) route to the desired benzophenanthridine but is probably not relevant to the cyclisation of the β-hydroxy esters.

In order to establish this route to the major antileukaemic alkaloids, it was necessary to show that the 12-hydroxy group could be removed. Two methods were tried; the first used thermal rearrangement of the dimethylthiocarbamate (8a) as a first step prior to desulphurisation (Scheme 2). The rearranged



Scheme 2. *Reagents and conditions*: i, dimethylthiocarbamoyl chloride, KOH; ii, heat.

product was obtained in poor yield, so the dehydroxylation was attempted instead *via* the tetrazolyl ether (Scheme 1). With palladium on charcoal, hydrogenation under pressure gave the de-hydroxybenzo[*c*]phenanthridines (6a) and (6c). The base (6c) is the required intermediate for a simple synthesis of the most potent antileukaemic alkaloid, fagaronine.

Experimental

Solutions of extracted products in organic solvents were dried over anhydrous magnesium sulphate before evaporation. IR spectra were recorded using potassium chloride discs or liquid films as appropriate. Elemental analyses which are quoted as hydrates are the results obtained after thorough drying and repeated combustion.

6,7-Dimethoxy-3-(3,4-dimethoxyphenyl)-1,2-dihydroisoquinolin-4(3H)-one (2a) *Hydrochloride*.—3,4-Dimethoxybenzylamine (16.7 g) in water (200 ml) was treated with dilute HCl until just acid. A solution of 3,4-dimethoxybenzaldehyde (16.6 g) in ethanol (100 ml) was added and the mixture heated on a steam bath for 30 min. Potassium cyanide (10 g) in water (50 ml) was added dropwise with vigorous stirring and the mixture stirred for a further 2 days, after which 2-(3,4-dimethoxybenzylamino)-2-(3,4-dimethoxyphenyl)acetonitrile (30.1 g, 88%) was collected by filtration and thoroughly washed with water. The amino nitrile (1a) had m.p. 55–57 °C (lit.,[2] 60–61 °C); IR, ^{1}H NMR and MS were in accord with those of authentic samples.

Cyclisation in hydrogen fluoride. The amino nitrile (27.4 g) in anhydrous hydrogen fluoride (150 ml) was stirred in a stoppered plastic bottle for 24 h. The reaction mixture was transferred to a plastic beaker and the HF allowed to evaporate. After 2 h the residue was diluted by the addition of crushed ice, stirred for 10 min and basified with solid sodium hydrogen carbonate while bubbling with nitrogen. The product was extracted with chloroform, the solution dried over anhydrous magnesium sulphate, diluted with ether and the isoquinoline hydrochloride (20.1 g, 66%) precipitated by addition of ethereal hydrogen chloride. The hydrochloride was sufficiently pure for further use and could be crystallised from water (m.p. 250–252 °C; lit.,[2] 255–256 °C).

Cyclisation in sulphuric acid. The aminonitrile (27.4 g) in chloroform (50 ml) was added to conc. sulphuric acid (100 ml) over a period of 10 min, the mixture stirred for 15 min, and the acid layer separated and diluted with crushed ice (*ca.* 500 g). The resulting greenish solution was stirred for 5 min and basified carefully with conc. ammonium hydroxide while cooling externally with an ice-bath and by addition of more ice. Nitrogen was bubbled through the reaction mixture during basification. The isoquinolinone was extracted with chloroform, and the solution washed with water and dried ($MgSO_4$). After filtration, excess ethereal hydrogen chloride was added to the chloroform solution and the crude isoquinolinone hydrochloride collected by filtration. Purification was effected by boiling with 96% ethanol which, on cooling, gave a white powder (16.4 g, 54%).

3-(4-Hydroxy-3-methoxyphenyl)-6,7-dimethoxy-1,2-dihydroisoquinolin-4(3H)-one (2d) *Hydrochloride*.—The method described above, using 4-isopropoxy-3-methoxybenzaldehyde in place of 3,4-dimethoxybenzaldehyde gave 2-(4-isopropoxy-3-methoxyphenyl)-2-(3,4-dimethoxybenzylamino)acetonitrile (1b), by chloroform extraction as an uncrystallisable gum in 97% yield; ν_{max} 2 240 (C≡N) and 3 320 cm^{-1} (NH); δ_H(300 MHz; $CDCl_3$) 1.35 (6 H, d, *J* 7 Hz, Me_2C), 1.90 (1 H, br s, exch., NH), 3.80 (9 H, s, OMe), 3.90 (2 H, s, CH_2N), 4.45 (1 H, sept, *J* 7 Hz, CHO), 4.65 (1 H, s, CH–N), and 6.7–7.1 (6 H, m, Ar); *m/z* 343.1784 (M^+ − 27, 18%. $C_{20}H_{25}NO_4$ requires 343.1783).

The aminonitrile (5 g) cyclised in anhydrous hydrogen fluoride, using the procedure described above, to give the *isoquinolinone hydrochloride*, m.p. 185–188 °C from water, in 31% yield (Found: C, 58.7; H, 5.5; N, 3.6. $C_{18}H_{20}ClNO_5$ requires C, 59.1; H, 5.5; N, 3.8%); ν_{max} 1 680 (C=O) and 3 460 cm^{-1} (OH); δ_H(300 MHz; [$^2H_6$]DMSO) 3.78 (3 H, s, OMe), 3.86 (3 H, s, OMe), 3.92 (3 H, s, OMe), 4.43 and 4.66 (2 H, AB system, *J* 15 Hz, 1-H), 5.46 (1 H, s, 3-H), 6.82–6.90 (2 H, m, Ar), 7.25 (1 H, br s, Ar), 7.26 (1 H, s, 8-H), and 7.47 (1 H, s, 5-H); *m/z* 329 (M^+, 27%), 327 (11), 300 (32), 178 (100), and 150 (15). The yield from sulphuric acid was 11%.

3-(4-*Ethoxy*-3-*methoxyphenyl*)-6,7-*dimethoxy*-1,2-*dihydroisoquinolin*-4(3H)-*one* (2c) *Hydrochloride*.—The aminonitrile synthesis described above, using 4-ethoxy-3-methoxybenzaldehyde, gave the desired product (1c) in 90% yield, m.p. 55–59 °C; ν_{max} 2 220 (C≡N) and 3 320 cm^{-1} (NH); δ_H(300 MHz; $CDCl_3$) 1.45 (3 H, t, *J* 7 Hz, CH_3C), 1.90 (1 H, br s, exch., NH), 3.85 (9 H, s, OMe), 3.90 (2 H, s, CH_2N), 4.05 (2 H, q, *J* 7 Hz, CH_2–O), 4.65 (1 H, s, CH–N), and 6.7–7.1 (6 H, m, Ar); *m/z* 329.1623 ($M^+ - 27$, 18%; $C_{19}H_{23}NO_4$ requires 329.1627) and 151 (100). The 2-(4-ethoxy-3-methoxyphenyl)-2-(3,4-dimethoxybenzylamino)acetonitrile thus obtained cyclised in anhydrous hydrogen fluoride, as described above, to give the *isoquinolinone hydrochloride*, m.p. 170–172 °C from aqueous ethanol, in 68% yield (Found: C, 58.6; H, 5.9; N, 3.4. $C_{20}H_{24}ClNO_5{\cdot}H_2O$ requires C, 58.3; H, 6.4; N, 3.4%); ν_{max} 1 670 cm^{-1} (C=O); δ_H(300 MHz; $CDCl_3$; free base) 1.30 (3 H, t, *J* 7 Hz, CH_3C), 2.50 (1 H, br s, exch., NH), 3.70 (3 H, s, OMe), 3.77 (3 H, s, OMe), 3.81 (3 H, s, OMe), 3.95 (2 H, q, *J* 7 Hz, CH_2–O), 3.95 (2 H, s, 1-H), 4.40 (1 H, s, 3-H), 6.65–7.0 (4 H, m, Ar), and 7.40 (1 H, s, 5-H); *m/z* 357 (M^+, 16%), 355 (25), 339 (100), 328 (16), 310 (60), 178 (41), and 150 (15). The yield from sulphuric acid was 29%.

4-*Ethoxycarbonylmethyl*-6,7-*dimethoxy*-3-(3,4-*dimethoxyphenyl*)-1,2,3,4-*tetrahydroisoquinolin*-4-*ol* (3a).—Zinc powder (98%) was washed with dry ether and dried under reduced pressure at 80 °C for 2 h. Ethyl bromoacetate was freshly distilled under reduced pressure. Dimethoxymethane was treated with sodium wire daily until no more reaction occurred and then distilled. Dioxane and benzene were dried over sodium wire and glassware was dried at 150 °C overnight. The isoquinolinone hydrochloride was converted into the free base, dried under reduced pressure for 3 h, and used fresh.

Zinc powder (19.22 g), dimethoxymethane (60 ml), and chlorotrimethylsilane (3 ml) were placed in a flask fitted with a dropping funnel, condenser, and drying tubes and stirred at room temperature for 15 min. The flask contents were heated to reflux and stirred while ethyl bromoacetate (30 ml) in dimethoxymethane (60 ml) was added dropwise: addition was halted after the addition of 10 ml of solution to ensure that the reaction started smoothly. The remaining solution was added slowly with the reaction temperature maintained at 90–100 °C, and the mixture stirred for 3 h. The heat was turned off and a solution of isoquinolinone (2a) (25.0 g) in dioxane (200 ml) added and the mixture stirred for 20 h. Saturated aqueous ammonium chloride (100 ml) and sufficient chloroform to form two layers were added and after filtration the organic layer was separated, washed with saturated aqueous ammonium chloride solution (2 × 30 ml) and water (2 × 20 ml), dried, and evaporated. The residue was redissolved in chloroform (50 ml) and added dropwise to light petroleum (b.p. 40–60 °C; 250 ml), to give the *tetrahydroisoquinolinol* (97%) as a white powder, m.p. 185–186 °C (from EtOAc) (Found: C, 63.9; H, 6.7; N, 3.6. $C_{23}H_{29}NO_7$ requires C, 64.0; H, 6.8; N, 3.3%); ν_{max} 1 715 (C=O) and 3 280 cm^{-1} (NH); δ_H(300 MHz; $CDCl_3$) 1.16 (3 H, t, *J* 7 Hz, CH_3C), 1.88 (2 H, br, OH and NH), 2.75 and 3.12 (2 H, AB system, *J* 15 Hz, 4-CH_2), 3.90 (6 H, s, OMe), 3.92 (6 H, s, OMe), 4.05 (2 H, q, *J* 7 Hz, O–CH_2), 4.04 and 4.26 (2 H, AB system, *J* 15 Hz, 1-H), 4.48 (1 H, s, 3-H), 6.60 (1 H, s, 8-H), 6.90 (1 H, d, *J* 9 Hz, Ar), 7.07 (1 H, dd, *J* 2 and 9 Hz, Ar), 7.08 (1 H, s, 5-H), and 7.12 (1 H, d, *J* 2 Hz, Ar); *m/z* 431 (M^+, 0.4%), 343 (17), 314 (19), 178 (40), 166 (100), and 150 (17).

4-*Ethoxycarbonylmethyl*-3-(4-*ethoxy*-3-*methoxyphenyl*)-6,7-*dimethoxy*-1,2,3,4-*tetrahydroisoquinolin*-4-*ol* (3c).—Using the above method, the isoquinolinone (2c) gave the *tetrahydroisoquinolinol* (86%), m.p. 169–170 °C (from EtOH) (Found: C, 64.5; H, 7.0; N, 3.1. $C_{24}H_{31}NO_7$ requires C, 64.7; H, 7.0; N, 3.1%); ν_{max} 1 715 (C=O) and 3 280 cm^{-1} (NH); δ_H(90 MHz; $CDCl_3$) 1.10 (3 H, t, *J* 7 Hz, ester Me), 1.44 (3 H, t, *J* 7 Hz, ether Me), 2.95 (2 H, br, OH and NH), 2.66 and 3.07 (2 H, AB system, *J* 15 Hz, 4-CH_2), 3.80 (3 H, s, OMe), 3.82 (3 H, s, OMe), 3.84 (3 H, s, OMe), 3.95 (2 H, q, *J* 7 Hz, ether CH_2), 4.05 (2 H, q, *J* 7 Hz, ester CH_2), 3.88 and 4.15 (2 H, AB system, *J* 15 Hz, 1-H), 4.38 (1 H, s, 3-H) 6.48 (1 H, s, 8-H), 6.78 (1 H, d, *J* 9 Hz, Ar), 6.95 (1 H, dd, *J* 2 and 9 Hz, Ar), 7.00 (1 H, s, 5-H), and 7.04 (1 H, d, *J* 2 Hz, Ar); *m/z* 445 (M^+, 0.2%), 357 (17), 328 (21), 180 (100), 178 (54), and 150 (24).

2,3,8,9-*Tetramethoxybenzo*[c]*phenanthridin*-12-*ol* (4a).—Concentrated sulphuric acid (40 ml) was added to the β-hydroxy ester (3a) (8.2 g), whereupon heat was evolved initially. After being stirred for 4 days the reaction mixture was poured onto ice and the yellow *benzo*[c]*phenanthridine sulphate* (4.6 g, 52%) collected by filtration. The free base crystallised from chloroform–ethanol and could be recrystallised from pyridine–ethanol, m.p. > 320 °C, softening at 204 °C (Found: C, 68.8; H, 5.3; N, 3.7. $C_{21}H_{19}NO_5$ requires C, 69.0; H, 5.2; N, 3.8%); λ_{max}(MeOH) 232 (ε 35 700 dm^3 mol^{-2} cm^{-1}), 250 (25 500), 286 (79 600), 340 (12 200), 358 (9 200), 380 (5 100) shifted to 294 (69 400), and 396 nm (12 200) with NaOH; δ_H(80 MHz; [$^2H_6$]DMSO) 3.97 (3 H, s, OMe), 4.00 (3 H, s, OMe), 4.03 (3 H, s, OMe), 4.10 (3 H, s, OMe), 7.63 (2 H, s, Ar), 7.73 (1 H, s, Ar), 7.80 (1 H, s, Ar), 8.60 (1 H, s, Ar), 9.13 (1 H, s, 6-H), and 10.38 (1 H, br, OH); *m/z* 365 (M^+, 100%), 364 (24), 350 (10), and 336 (12).

2-*Ethoxy*-3,8,9-*trimethoxybenzo*[c]*phenanthridin*-12-*ol* (4c) *and* 3,8,9-*Trimethoxybenzo*[c]*phenanthridine*-2,12-*diol* (4d).—Concentrated sulphuric acid (60 ml) was added to the β-hydroxy ester (3c) (12.0 g). The mixture was stirred for 24 h, diluted with ice, basified and extracted to give the crude benzo[*c*]phenanthridine. Chromatography on neutral alumina, eluting with chloroform–ethanol (9:1) gave the pure 2-*ethoxybenzo*[c]*phenanthridine* (4c) (70%) m.p. > 320 °C (from $CHCl_3$–EtOH) (Found: C, 67.9; H, 5.6; N, 3.5. $C_{22}H_{21}NO_5{\cdot}0.5H_2O$ requires C, 68.0; H, 5.7; N, 3.6%); λ_{max}(MeOH) 233 (ε 25 000 dm^3 mol^{-1} cm^{-1}), 251 (19 400), 287 (58 900), 342 (8 900), 359 (7 800), 378 (4 400) shifted to 294 (52 800), and 396 nm (9 400) with NaOH; δ_H(90 MHz; [$^2H_6$]DMSO) 1.40 (3 H, t, *J* 7 Hz, CH_3C), 3.97 (3 H, s, OMe), 4.03 (3 H, s, OMe), 4.07 (3 H, s, OMe), 4.20 (2 H, q, *J* 7 Hz, O–CH_2), 7.63 (2 H, s, Ar), 7.73 (1 H, s, Ar), 7.80 (1 H, s, Ar), 8.62 (1 H, s, Ar), 9.13 (1 H, s, 6-H), and 10.36 (1 H, br, OH); *m/z* 379 (M^+, 100%), 378 (13), 350 (38), and 322 (14).

If exposure to sulphuric acid was extended to 2 days, chromatography as above gave first the 2-ethoxybenzo[*c*]phenanthridine (4c) (21%). Elution with chloroform–ethanol (1:4) then gave the *benzo*[c]*phenanthridine*-2,12-*diol* (4d) (14%), m.p. 225–227 °C (from $CHCl_3$–EtOH) (Found: C, 66.7; H, 4.8; N, 3.8. $C_{20}H_{17}NO_5{\cdot}0.5H_2O$ requires C, 66.7; H, 5.1; N, 3.9%); λ_{max}(MeOH) 235 (ε 31 500 dm^3 mol^{-1} cm^{-1}), 251 (20 700), 289 (53 200), 343 (8 100), 356 (6 300), 380 (4 000) shifted to 296 (39 200), and 359 nm (9 000) with NaOH; δ_H 3.97 (3 H, s, OMe), 4.03 (3 H, s, OMe), 4.07 (3 H, s, OMe), 7.60 (1 H, s, Ar), 7.63 (1 H, s, Ar), 7.70 (1 H, s, Ar), 7.80 (1 H, s, Ar), 7.80 (1 H, s, Ar), 8.60 (1 H, s, Ar), 9.13 (1 H, s, 6-H), and 10.36 (2 H, br, OH); *m/z* 351 (M^+, 100%), 350 (29), and 308 (10).

3-(3,4-*Dimethoxyphenyl*)-4-*ethoxycarbonylmethyl*-6,7-*dimethoxyisoquinoline* (7a).—The β-hydroxy ester (3a) (2.06 g) was heated under reflux in ethanolic hydrogen chloride (55 ml) for 3 h. The mixture was concentrated to one-fifth volume and the crystalline isoquinoline hydrochloride (1.2 g, 56%; ν_{max} 1 720 cm^{-1}) collected by filtration. The *isoquinoline* was obtained by basification and extraction with chloroform, m.p. 123–125 °C

[from EtOAc–light petroleum (b.p. 60–80 °C)] (Found: C, 67.0; H, 6.1; N, 3.4. $C_{23}H_{25}NO_6$ requires C, 67.1; H, 6.1; N, 3.4%); *m/z* 411 (M^+, 100%), 396 (17), 382 (6), and 338 (43).

When the isoquinoline hydrochloride (0.6 g) was dissolved in concentrated sulphuric acid (4 ml), stirred and monitored by TLC daily, no benzo[*c*]phenanthridine (**4a**) was detected in 22 days.

4-*Carboxymethyl*-3-(3,4-*dimethoxyphenyl*)-6,7-*dimethoxyisoquinoline Hydrochloride* (**7b**) *and Its Conversion into* 2,3,8,9-*tetramethoxybenzo*[c]*phenanthridin*-12-*ol* (**4a**).—The ester hydrochloride (**7a**) (0.3 g) was boiled under reflux in 5M hydrochloric acid for 5 h. Evaporation to dryness gave the 4-*carboxymethylisoquinoline hydrochloride* (0.22 g, 79%). The analytical sample was crystallised from ethanol–ether, m.p. 169–174 °C (Found: C, 58.9; H, 5.2; N, 3.7. $C_{21}H_{21}NO_6 \cdot 0.5H_2O$ requires C, 58.8; H, 5.4; N, 3.3%); ν_{max} 1 700 cm^{-1}.

The carboxymethyl isoquinoline hydrochloride (0.14 g) was added to concentrated sulphuric acid (5 ml) and stirred overnight. Dilution with ice, basification with sodium carbonate and extraction with chloroform gave 2,3,8,9-tetramethoxybenzo[*c*]phenanthridin-12-ol (80 mg, 66%).

12-(*Dimethylthiocarbamoyloxy*)-2,3,8,9-*tetramethoxybenzo*[c]*phenanthridine* (**8a**).—The benzo[*c*]phenanthridin-12-ol (**4a**), (1.0 g) was dissolved in aqueous potassium hydroxide (10%; 15 ml). Tetrahydrofuran (3 ml) was added and the solution cooled to below 10 °C with an ice-bath. Dimethylthiocarbamoyl chloride (1.7 g) in tetrahydrofuran (5 ml) was added dropwise, with cooling. Stirring was continued for 15 min after which the mixture was diluted with water (20 ml) and the crude product collected by filtration. Crystallisation ($CHCl_3$–MeOH) gave the *thiocarbamate* (0.41 g, 42%), m.p. 274–276 °C (Found: C, 61.3; H, 5.4; N, 5.8. $C_{24}H_{24}N_2O_5 \cdot H_2O$ requires C, 61.3; H, 5.6; N, 6.0%); δ_H 3.65 (6 H, s, NMe_2), 4.05 (3 H, s, OMe), 4.10 (3 H, s, OMe), 4.15 (3 H, s, OMe), 4.20 (3 H, s, OMe), 7.18 (1 H, s, Ar), 7.38 (1 H, s, Ar), 7.78 (1 H, s, Ar), 8.00 (1 H, s, Ar), 8.75 (1 H, s, Ar), and 9.23 (1 H, s, 6-H); *m/z* 452 (M^+, 61%), 365 (14), 88 (100), and 72 (81).

12-(*Dimethylcarbamoylthio*)-2,3,8,9-*tetramethoxybenzo*[c]*phenanthridine*.—The *O*-arylthiocarbamate described above (**8a**) (42 mg) in digol (1 ml) was heated at 250 °C for 1.5 h, cooled, and refrigerated for 1 week. The supernatent was decanted and the residue triturated with ether. The solid was collected by filtration and crystallised (EtOH) to give the *benzophenanthridine* (10 mg, 24%), m.p. 235–240 °C, recrystallising at 222 °C. There was insufficient material for microanalysis, but IR, ^1H NMR, and MS confirmed the structure: ν_{max}(KCl) 1 660 cm^{-1} (C=O); δ_H 3.18 (6 H, br, NMe_2), 4.08 (6 H, s, OMe), 4.15 (3 H, s, OMe), 4.18 (3 H, s, OMe), 7.35 (1 H, s, Ar), 7.73 (1 H, s, Ar), 7.83 (1 H, s, Ar), 8.60 (1 H, s, Ar), 8.73 (1 H, s, Ar), and 9.23 (1 H, s, 6-H); *m/z* 452.1416 (68%; $C_{24}H_{24}N_2O_5S$ requires 452.1406), 380 (16), and 72 (100).

2,3,8,9-*Tetramethoxy*-12-(1-*phenyl*-1H-*tetrazol*-5-*yloxy*)*benzo*[c]*phenanthridine* (**5a**).—A mixture of dry acetone (20 ml), anhydrous potassium carbonate (3 g) and the benzo[*c*]phenanthridinol (**4a**) sulphate (2.48 g) was stirred and warmed for 15 min. 5-Chloro-1-phenyl-1*H*-tetrazole (1.4 g) was added and the mixture heated under reflux for 24 h. After cooling and addition of aqueous sodium hydroxide (5%; 20 ml) the solid product was collected by filtration and washed with water. The *tetrazolyl ether* crystallised from MeOH–$CHCl_3$ as long white needles (2.033 g, 75%), m.p. 258–260 °C (decomp.) (Found: C, 64.9; H, 4.3; N, 13.3. $C_{28}H_{23}N_5O_5 \cdot 0.5H_2O$ requires C, 64.9; H, 4.7; N, 13.5%); *m/z* 509.1703 (M^+; $C_{28}H_{23}N_5O_5$ requires 509.1699); δ_H(80 MHz; $CDCl_3$), 3.85 (3 H, s, OMe), 4.09 (3 H, s, OMe), 4.14 (3 H, s, OMe), 4.17 (3 H, s, OMe), 7.24 (1 H, s, Ar), 7.36 (1 H, s, Ar), 7.59–7.75 (4 H, s, and m, Ar), 7.91–7.98 (2 H, m, Ar), 8.73 (2 H, s, Ar), and 9.19 (1 H, s, 6-H).

2,3,8,9-*Tetramethoxybenzo*[c]*phenanthridine* (**6a**).—The above tetrazolyl ether (**5a**) (2.0 g) in dioxane (100 ml) was hydrogenated over palladium-on-charcoal (5%; 1.0 g) at 60 °C for 24 h., at 10 atm. The cooled mixture was filtered and the solid collected, placed on a short column of silica gel and eluted with $CHCl_3$–EtOH (9:1) to give the *benzophenanthridine* (0.9 g, 66%). A sample recrystallised from pyridine had m.p. 307–310 °C (lit.,[8] m.p. varies from 299–301 °C to 306–308 °C); δ_H 4.08 (3 H, s, OMe), 4.09 (3 H, s, OMe), 4.15 (3 H, s, OMe), 4.20 (3 H, s, OMe), 7.29 (1 H, s, Ar), 7.37 (1 H, s, Ar), 7.85 (1 H, s, *J* 9 Hz, Ar), 7.90 (1 H, s, Ar), 8.29 (1 H, d, *J* 9 Hz, Ar), 8.74 (1 H, s, Ar), and 9.24 (1 H, s, Ar), and 9.24 (1 H, s, 6-H); *m/z* 349 (M^+, 100%).

2-*Ethoxy*-3,8,9-*trimethoxy*-12-(1-*phenyl*-1H-5-*tetrazol*-5-*yloxy*)*benzo*[c]*phenanthridine* (**5c**).—A mixture of the benzo[*c*]phenanthridinol (**4c**) 0.8 g dry dimethylformamide (10 ml), and potassium t-butoxide (0.33 g) was stirred under nitrogen until the phenol dissolved (3 min). 5-Chloro-1-phenyl-1*H*-tetrazole (0.5 g) was added and the mixture stirred under nitrogen for 20 min. The cloudy solution was poured onto ice and the *tetrazolyl ether* (0.7 g, 64%) collected by filtration. The analytical sample crystallised as long needles, m.p. 260–266 °C (MeOH–$CHCl_3$) (Found: C, 66.2; H, 5.0; N, 13.4. $C_{29}H_{25}N_5O_5$ requires C, 66.5; H, 4.8; N, 13.4%); *m/z* 523 (M^+, 8%), 495 (49), 453 (32), and 379 (100); δ_H(80 MHz; CF_3COOD) 1.39 (3 H, t, *J* 7.8 Hz, CH_3C), 4.14 (6 H, s, OMe), 4.25 (3 H, s, OMe), 4.08 (2 H, q, *J* 7.8 Hz, CH_2C), 7.45 (1 H, s, Ar), 7.61–7.85 (6 H, m and s, Ar), 8.08 (1 H, s, Ar), 8.20 (1 H, s, Ar). 8.84 (1 H, s, Ar), and 9.42 (1 H, s, 6-H).

2-*Ethoxy*-3,8,9-*trimethoxybenzo*[c]*phenanthridine* (**6c**).—The above tetrazolyl ether (**5c**) (0.56 g) was hydrogenated as described to give the *benzo*[c]*phenanthridine* (155 mg, 40%), m.p. 271–274 °C, recrystallising at 233–238 °C (from $CHCl_3$–MeOH); δ_H 1.60 (3 H, t, *J* 8 Hz, CH_3C), 4.10 (3 H, s, OMe), 4.17 (3 H, s, OMe), 4.20 (3 H, s, OMe), 4.33 (2 H, q, *J* 8 Hz, CH_2O), 7.30 (1 H, s, Ar), 7.40 (1 H, s, Ar), 7.87 (1 H, d, *J* 9 Hz, Ar), 7.93 (1 H, s, Ar), 8.33, (1 H, d, *J* 9 Hz, Ar), 8.77 (1 H, s, Ar), and 9.27 (1 H, s, 6-H); *m/z* 363.1467 (100%; $C_{22}H_{21}NO_4$ requires 363.1470), 348 (6.1), 334 (40.6), and 318 (5.7).

Acknowledgements

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References

1 M. Suffness and G. A. Cordell in 'The Alkaloids,' ed. A. Brossi, Academic Press, New York, 1985, vol. 25, p. 178.
2 D. N. Harcourt and R. D. Waigh, *J. Chem. Soc. C*, 1971, 967.
3 G. A. Cordell and N. R. Farnsworth, *Heterocycles*, 1976, 4, 393.
4 C. Feugeas, *Bull. Soc. Chim. Fr.*, 1963, 2568; J. C. Stowell, *J. Org. Chem.*, 1976, 41, 560.
5 J. Cure and M. Gaudemar, *Bull. Soc. Chim. Fr.*, 1969, 2471; M. W. Rathke, *Org. React.* 1975, 22, 423.
6 G. Picotin and P. Miginiac, *J. Org. Chem.*, 1987, 52, 4796.
7 R. H. F. Manske in 'The Alkaloids,' ed. R. H. F. Manske, Academic Press, New York, 1954, vol. 4, p. 256.
8 A. S. Bailey, R. Robinson, and R. S. Staunton, *J. Chem. Soc.*, 1950, 2277; S. F. Dyke and M. Sainsbury, *Tetrahedron*, 1967, 23, 3161; S. F. Dyke, M. Sainsbury, and B. J. Moon, *ibid.*, 1968, 24, 1467.

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Cyclisation of Benzylaminonitriles. Part 8.† Anhydrous Hydrogen Fluoride as Cyclising Agent

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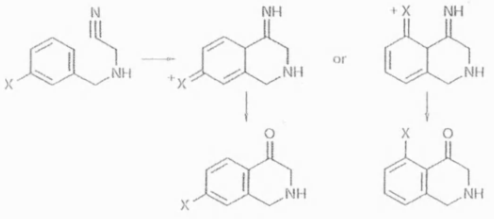
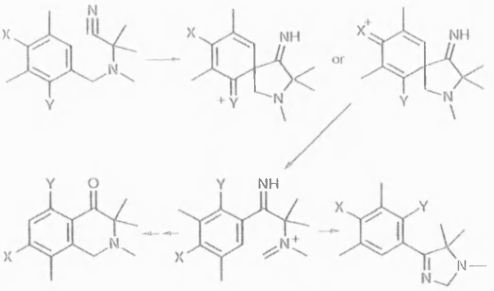
We report that the preferred cyclisation pathway for benzylaminonitriles in anhydrous HF is through a spiro intermediate, as it is in sulphuric acid. However, unlike sulphuric acid, HF can be used in the cyclisation of *N*-alkyl benzylaminonitriles to give *N*-alkylisoquinolinones and also succeeds with methylenedioxy-substituted amines which decompose in sulphuric acid.



Scheme 1 X = OMe or SMe

The cyclisation of benzylaminonitriles has been shown to be a useful route to isoquinolinones, imidazolines and benzazepines.[1] It may proceed by direct attack of the protonated nitrile on the benzene ring (Scheme 1), but where possible a preferred route is through a spiro intermediate (Scheme 2). Almost all the work on the reaction in the last 20 years has involved the use of concentrated sulphuric acid as solvent and catalyst; many other acids have been tried but with minimal success. In many cases the use of sulphuric acid gives perfectly acceptable results, but in some instances problems arise through sulphonation, dealkylation or diversion to quite unexpected products. All these problems are avoided by the use of anhydrous hydrogen fluoride as solvent and cyclisation catalyst.

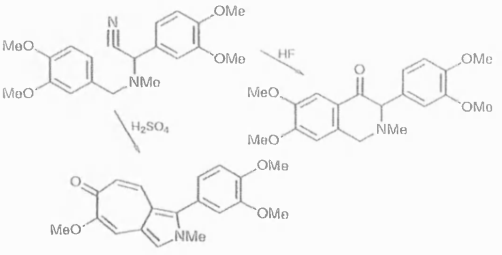


Scheme 2 X or Y = OMe or SMe

The use of anhydrous hydrogen fluoride (HF) for intramolecular acylations was pioneered by Fieser in the late 1930's. He obtained very high yields[2] in reactions where a carboxylic acid attacked a benzene ring in solution in HF. The latter was then allowed to evaporate, leaving the cyclic ketone. Despite the precautions which have to be taken in the use of HF, particularly to avoid inhalation or skin contact and to be aware of the corrosive effect on building materials, it remains a highly effective reagent which has been employed regularly for cyclisations in the years since Fieser's work.[3]

Previous work has demonstrated that HF can result in improved yields of isoquinolinones from cyclisation of benzylaminonitriles, most importantly by avoiding sulphonation[4] but also through a reduced susceptibility to *O*-dealkylation.[5] There is an important benefit, also, in the very uncomplicated work-up required with HF, which can be allowed to evaporate or blown off with nitrogen.

In view of the different mechanisms which operate in sulphuric acid it was important to determine the mechanism(s) of cyclisation in HF. The earlier work had shown that direct attack by the protonated nitrile on the benzene ring was possible,[4] as in Scheme 1, but other reported cyclisations[5] could have proceeded by either route. From cyclisation of **1a** it is apparent that the spiro rearrangement (Scheme 2) is also the preferred route in HF. The product **2a** was obtained in low yield owing to a strong tendency to oxidise in air, as has been observed previously with analogous isoquinolinones.[6] The chemical shift of the aromatic protons of the isoquinolinone B ring (7.31 and 7.49 ppm) suggested that rearrangement had occurred, since a 5-proton in a 7,8-dimethoxyisoquinolin-4-one would be expected to resonate at *ca.* 7.9 ppm, and this was confirmed by observation of nuclear Overhauser effects between H-8 (7.31 ppm) and the protons at position 1. A preference for the spiro route was confirmed by cyclisation of **1b** to give the imidazoline **3b** and by ring closure of the trimethoxy derivative **1c** which gave both the imidazoline **3c** and the isoquinolinone **2c**.

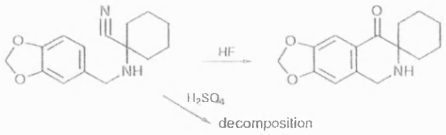


Scheme 3

With two types of aminonitrile the effects of sulphuric acid are peculiar, yet HF gives 'normal' results which significantly extend the scope of the reaction. These are summarised in Schemes 3 and 4. Use of sulphuric acid resulted in the production of a non-basic, strongly coloured cycloheptapyrrole[8] from the aminonitrile **1d** as shown in Scheme 3. This was a major product in the presence of an *N*-alkyl substituent and meant that *N*-alkylisoquinolinones were not directly available by this route. In HF the reaction proceeded normally, giving excellent yields of the isoquinolinones **2d** and **2e** from the aminonitriles **1d** and **1e**.

* To receive any correspondence.

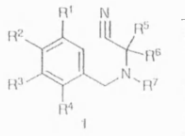
† For Part 7, see J. P. Gavin and R. D. Waigh, *J. Chem. Soc., Perkin Trans. 1*, 1990, 503.



Scheme 4

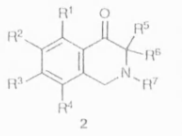
Concentrated sulphuric acid has been used as a reagent for the detection of methylenedioxy groups through liberation of formaldehyde.[9] It is not surprising, therefore, that treatment of methylenedioxy-substituted aminonitriles with sulphuric acid does not give the desired isoquinolinones. In contrast (Scheme 4), the cyclisation of **1f** and **1g** succeeded in HF, giving moderate yields of the 6,7-methylenedioxyisoquinolinones **2f** and **2g**. NMR spectra showed the presence of the 7,8-methylenedioxy isomer from both **1f** and **1g** in *ca.* 7% yield in the crude product. No attempt was made to isolate the minor product.

We thank the S.E.R.C. for studentship support of M. R. E. and J. P. G. and the Nigerian Government for support of T. A. O.



1

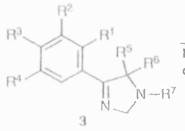
	R^1	R^2	R^3	R^4	R^5	R^6	R^7
a	H	H	MeO	MeO	DMP	H	H
b	H	MeS	H	H	Me	Me	Me
c	MeO	MeO	H	MeO	-$[CH_2]_5$-		H
d	H	MeO	MeO	H	DMP	H	Me
e	H	MeO	MeO	H	EMP	H	Me
f	H	-O-CH_2-O-		H	-$[CH_2]_5$-		H
g	H	-O-CH_2-O-		H	Me	Me	H



2

	R^1	R^2	R^3	R^4	R^5	R^6	R^7
a	MeO	MeO	H	H	DMP	H	H
c	MeO	H	MeO	MeO	-$[CH_2]_5$-		H
d	H	MeO	MeO	H	DMP	H	Me
e	H	MeO	MeO	H	EMP	H	Me
f	H	-O-CH_2-O-		H	-$[CH_2]_5$-		H
g	H	-O-CH_2-O-		H	Me	Me	H

DMP = 3,4-dimethoxyphenyl
EMP = 4-ethoxy-3-methoxyphenyl



3

	R^1	R^2	R^3	R^4	R^5	R^6	R^7
b	H	H	MeS	H	Me	Me	Me
c	MeO	H	MeO	MeO	-$[CH_2]_5$-		H

Techniques used: IR, ^1H NMR

References: 10

Schemes: 4

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References cited in this synopsis

1 D. N. Harcourt, F. Hussain, N. Taylor and M. Nasir, *J. Chem. Soc., Perkin Trans. 1*, 1986, 1329, and references cited therein.
2 L. F. Fieser and E. B. Hershberg, *J. Am. Chem. Soc.*, 1939, **61**, 1272.
3 For example, W. C. Johnson and E. J. Matthews, *J. Am. Chem. Soc.*, 1944, **66**, 211; G. H. Daub, M. E. Ackerman and F. Newton Hayes, *J. Org. Chem.*, 1973, **38**, 828; M. S. Newman and V. K. Khanna, *J. Org. Chem.*, 1986, **51**, 1921.
4 M. R. Euerby and R. D. Waigh, *J. Chem. Res.*, 1987, (*S*) 36; (*M*) 0501; (*S*) 38; (*M*) 0527.
5 T. A. Olugbade, R. D. Waigh and S. P. Mackay, *J. Chem. Soc., Perkin Trans. 1*, 1990, 2657.
6 D. N. Harcourt and R. D. Waigh, *J. Chem. Soc. C*, 967, 1971.
8 R. D. Waigh, *J. Chem. Soc., Chem. Commun.*, 1980, 1164.
9 O. R. Hansen, *Acta Chem. Scand.*, 1953, 7, 1125.

Hz), 1.41-1.60 [overlapping C_{8a}-H and $(C\underline{H}_2\text{-}CH_3)_2$, 5 H], 0.90 (s, 3 H, C_1-CH_3), 0.88 [overlapping t's, 6 H, $(CH_2\text{-}C\underline{H}_3)_2$] ; chemical shifts were determined from the ^{1}H COSY spectrum; **8a** ^{1}H NMR ($CDCl_3$) δ 7.22 (t, 1 H, m-Ar H), 6.78 (m, 3 H o- and p-Ar H's), 5.76 (m, 1 H, C_{10}-H), 5.20 (m, 2 H, C_{11}-H's), 4.73 (d, 1 H, C_2-H, $J_{2a\text{-}8a}$ = 1.0 Hz), 4.17 (ddd, 1 H, C_4-H, $J_{4\text{-}5}$ = 1.1, $J_{4\text{-}12}$ = 6.6 and $J_{4\text{-}12'}$ = 8.7 Hz), 3.79 (s, 3 H, OCH_3), 3.32 [p, 1 H, $C\underline{H}(Et)_2$, J = 5.8 Hz], ~3.17 (dd, 1 H, $ArCH_{12}\underline{H}_{12'}$, $J_{4\text{-}12'}$ = 8.7 and $J_{12\text{-}12'}$ =14.2 Hz), 2.97(ddd, 1 H, C_{7a}-H, $J_{7a\text{-}8e}$ = 9.0, $J_{7a\text{-}8a}$ = 12 and $J_{7a\text{-}7e}$ = 16 Hz), ~2.85 (dd, 1 H, $ArC\underline{H}_{12}H_{12'}$ $J_{4\text{-}12}$ = 6.6 and $J_{12\text{-}12'}$ =14.2 Hz), 2.76 (br d, 1 H, C_9-H, $J_{9\text{-}10}$= 8 Hz), 2.38 (br s, 1 H, C_5-H), 2.30 (dd, 1 H, C_{7e}-H, dd, $J_{7e\text{-}8a}$= 6.2 and $J_{7a\text{-}7e}$ = 16 Hz), 2.20 (dd, 1 H, C_{8e}-H, $J_{7a\text{-}8e}$ = 9 and $J_{8a\text{-}8e}$ =12 Hz], 1.35-1.54 [overlapping C_{8a}-H and $(C\underline{H}_2\text{-}CH_3)_2$, 5 H], 0.96 (s, 3 H, C_1-CH_3), 0.89 (t, 3 H, CH_2-$C\underline{H}_3$, J = 7 Hz), 0.78 (t, 3 H, CH_2-$C\underline{H}_3$, J = 7 Hz) ; chemical shifts were determined from the ^{1}H COSY spectrum; **8b** ^{1}H NMR ($CDCl_3$) δ 7.20 (t, 1 H, m-Ar H), 6.70 (m, 3 H o- and p-Ar H's), 5.73 (m, 1 H, C_{10}-H), 5.19 (m, 2 H, C_{11}-H's), 4.73 (s, 1 H, C_2-H), 3.98 (dd, 1 H, C_4-H, $J_{4\text{-}12}$ = 5.0 and $J_{4\text{-}12'}$ = 10 Hz), 3.77 (s, 3 H, OCH_3), 3.75 [m, 1 H, $C\underline{H}(Et)_2$], ~3.3 (dd, 1 H, $ArCH_{12}\underline{H}_{12'}$, $J_{4\text{-}12'}$ = 10.3 and $J_{12\text{-}12'}$ = 14 Hz), ~3.2 (dd, 1 H, $ArC\underline{H}_{12}H_{12'}$, $J_{4\text{-}12}$ = 5, and $J_{12\text{-}12'}$ = 14 Hz), 3.22 (d, 1 H, C_9-H, $J_{9\text{-}10}$ = 8 Hz), 2.84 (ddd, 1 H, C_{7a}-H, $J_{7a\text{-}8e}$ = 9.4 , $J_{7a\text{-}8a}$ = 11.9 and $J_{7a\text{-}7e}$ = 16.3 Hz), 2.37 (br s, 1 H, C_5-H), 2.26 (dd, 1 H, C_{7e}-H, $J_{7e\text{-}8a}$ = 6 and, $J_{7a\text{-}7e}$ = 16 Hz), 1.50-1.93 (overlapping C_{8a}-H, C_{8e}-H and $(C\underline{H}_2\text{-}CH_3)_2$, 6 H], 0.95 (s, 3 H, C_1-CH_3), 0.89 [t, 6 H, $(CH_2\text{-}C\underline{H}_3)_2$], J = 7 Hz] ; chemical shifts were determined from the ^{1}H COSY spectrum.

14. Satisfactory analytical and high-resolution mass spectral data were consistent with the proposed structures.

(Received in US 26 October, 1990)

SYNTHETIC COMMUNICATIONS, 21(1), 43-48 (1991)

IMPROVED REGIOSELECTIVITY OF LITHIATION OF METHOXYBENZALDEHYDES

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Abstract: Protection of the aldehyde moiety as a N,N'-diisopropylimidazolidine allows improved preferential lithiation *ortho* to the methoxy group of 4- methoxybenzaldehyde or in the 4-position of 3-methoxybenzaldehyde. Reaction with a suitable electrophile, such as dimethyldisulphide, gives good yields of 3-substituted-4-methoxybenzaldehydes or moderate yields of 4-substituted-3-methoxybenzaldehydes as appropriate.

Lithiation of methoxybenzaldehydes presents a problem, in that normal protection strategies such as acetal formation tend to induce lithiation *ortho* to the protecting group,[1] in competition with lithiation *ortho* to the methoxy substituent, where this is required. Euerby and co-workers[2] have shown that the choice of protecting group and of lithiating reagent allows some regioselectivity, *t*-butyllithium tending to result in a preference for reaction *ortho* to a N,N'-dimethylimidazoline protecting group, while *n*-butyllithium tends to favour lithiation *ortho* to the methoxy, particularly in the presence of N,N,N',N'-tetramethylethylenediamine. With a requirement for efficient

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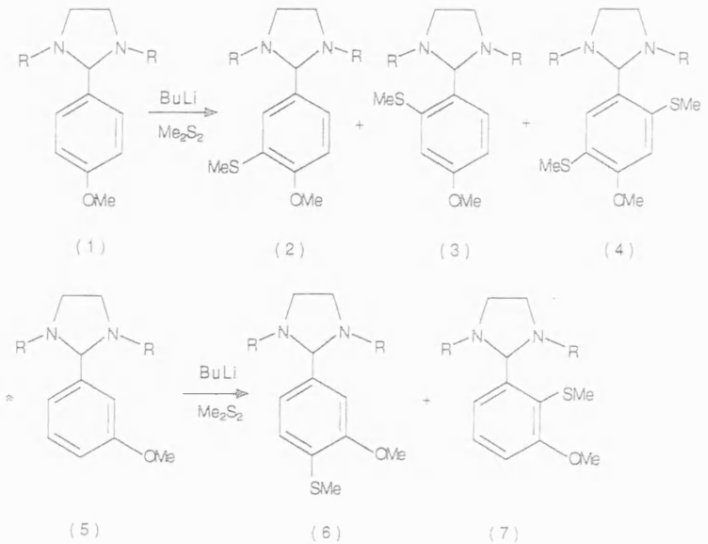
syntheses of both 3-methoxy-4-methylthio and 4-methoxy-3-methylthio benzaldehyde we set out to improve the preference for lithiation *ortho* to the methoxy group, by increasing the bulk of the imidazolidine N-substituents.



(a) R = Me, (b) R = Et, (c) R = iPr, (d) R = CH_2CMe_3

The previous work[2] has shown that the presence of TMEDA in the reaction mixture improves selectivity towards lithiation at sterically unobstructed sites, presumably by forming an intermediate complex which increases the effective steric bulk of the lithium. With one mole of the *p*-methoxyimidazolidine (1a) and two moles of all the other reagents, Euerby and co-workers reported[2] that the required intermediate (2a) was obtained

as 84% of the mixture of products, with 8% of each of the unwanted methylthiolated derivatives (3a) and (4a). In our hands the results were similar, although the relative proportions of (3a) and (4a) were slightly different (Table) and purification resulted in substantial losses. When the N-substituents of the protecting group were changed to ethyl (1b), but otherwise using the same methodology as the previous workers[2], the regioselectivity was substantially improved, with the required isomer (2b) obtained as 95% of the product mixture. A further increase in bulk to isopropyl (1c) virtually eliminated the unwanted products and gave 97% of the reaction product as the required 4-methoxy-3-methylthiophenylimidazolidine (2c). Both N,N'-diethyl and N,N'-diisopropyl 1,2-diaminoethane are readily available from commercial sources. Deprotection of the imidazolidines proceeded in dilute acid[2] to give almost quantitative yields of the aldehydes; from (2c) the aldehyde was sufficiently pure for further reactions with no need for either distillation or crystallisation.

There is a particular problem with the 3-methoxy isomer, since lithiation between the two substituents offers the possibility of stabilisation from both sides. The previous workers had reported[2] that the required 4-substituted aldehyde was formed as only 68% of the product when *m*-methoxybenzaldehyde was the starting material and reaction conditions were as described above, with two moles of each of TMEDA, butyllithium and dimethyldisulphide. From the N,N'-dimethylimidazolidine (5a) we obtained very similar results (Table). With the N,N'-diethyl analogue (5b) the proportion of the desired isomer was increased to 79% and the N,N'-diisopropyl compound (5c) gave a further increase to 82%. A change to 4

Table

Starting imidazolidine	Product ratios (%)[a]		
	(2)	(3)	(4)
(1a)	86	10	4
(1b)	95	2.5	2.5
(1c)	97	1.5	1.5
	(6)	(7)	
(5a)	72	28	
(5b)	79	21	
(5c)	86	14	
(5d)	70	30	

[a]All with a ratio of protected benzaldehyde: TMEDA: butyllithium: dimethyldisulphide of 1:2:2:2 except for (5c) where the ratio was 1:4:2:2. Ratios with less TMEDA, such as 1:1:2:2 gave consistently poorer yields of the desired products.

moles of TMEDA made a further slight improvement, to 86%. In making these changes the amount of unwanted isomer (7) was reduced from *ca* 30% to 14%. After hydrolysis of the imidazolidine (6c), 3-methoxy-4-methylthiobenzaldehyde could be obtained in a pure form by flash column chromatography in an overall yield of 57%.

In an attempt to improve the regioselectivity further, we investigated the use of the N,N'-dineopentyl analogue (5d). The required diamine is not

commercially available, unlike the other diamines described above. The synthesis was achieved by means of reduction with diborane of the N,N'-dipivalamide of ethylene diamine, after which the imidazolidine (5d) was produced with slightly greater difficulty than required for the analogues with smaller protecting groups. Unfortunately, the regioselectivity obtained was no better than with the N,N'-dimethyl compound (5a). With molecular modelling it is possible to show that the important region for increased steric bulk is immediately around the imidazoline nitrogens and not one carbon further removed. The modelling shows that the effect of increased bulk in the region of the imidazolidine nitrogens is to cause the aromatic ring to rotate, preferring to adopt conformations in which the planes of the two rings are approximately orthogonal. This rotational preference is reflected in the chemical shift of the proton between the two nitrogens, which in the series (1a)-(1b)-(1c) changes from δ3.19 - 3.43 - 4.15 and in the series (5a)-(5b)-(5c)-(5d) from δ3.23 - 3.48 - 4.19 - 3.57, approximately in parallel with the regioselectivity induced.

As a brief test of the generality of these findings we have carried out reactions with methyl iodide as the electrophile, on the lithiation product of both (1) and (5). In both cases the reaction conditions were as for the majority of the reactions in the Table, i.e. 1:2:2:2 as defined there, and very similar results were obtained.

Acknowledgements

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References

1. H.P. Plaumann, B.A. Keay and R. Rodrigo, *Tetrahedron Lett.*, 1979, **51**, 4921
2. F.A. Bevan, M.R. Euerby and S.J. Qureshi, *J. Chem. Research (S)*, 1989, 116
3. H.C. Brown and P. Heim, *J. Org. Chem.*, 1973, **38**, 912

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SYNTHETIC COMMUNICATIONS, 21(1), 49-61 (1991)

SYNTHESIS OF A NOVEL HEXA CYCLIC BENZIMIDAZO[1,2-c]INDAZOLO-[2,3-a]QUINAZOLINE

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ABSTRACT : Two alternate synthetic routes are described for the synthesis of the hitherto unknown benzimidazo[1,2-c]indazolo[2,3-a]quinazoline (3). While dehydrative cyclisation of 2-[2-(2-azidobenzoylamino)-phenyl]benzimidazole (2) in polyphosphate ester gave 3 in low yield, very good yields were obtained in triethyl phosphite reductive cyclisation of 6-(2-nitrophenyl)benzimidazo[1,2-c]quinazolines (8).

Quinazolines annelated to benzimidazoles and to indazoles were extensively studied for anti-inflammatory activity[1,2]. The paucity of literature on a system incorporating these three heterocycles has encouraged us to work out a facile synthesis of the novel hexacyclic system benzimidazo[1,2-c]indazolo[2,3-a]quinazoline, and we present here two alternate routes. One of the methods involves the polyphosphate ester dehydrative cyclisation of 2-[2-(2-azidobenzoylamino)phenyl]benzimidazole (2) and the other is by triethyl phosphite reductive cyclisation of 6-(2-nitrophenyl)benzimidazo[1,2-c]quinazoline (8). Recently, we reported

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Bull Soc Chim Fr (1994) 131, 718-722

Communication

High-yielding total synthesis of fagaronine chloride

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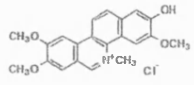
(received 30 March 1994, accepted 20 June 1994)

Summary – A versatile method for the synthesis of the benzo[c]phenanthridine nucleus was developed and applied to the preparation of fagaronine chloride. This method provided an overall yield of 14%, superior to other existing strategies, from a linear route comprising of nine steps and readily available reagents.

benzo[c]phenanthridine alkaloid / fagaronine / aminonitrile Reformatski reaction / phenolic dehydroxylation

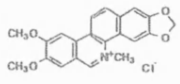
Introduction

Fully aromatized quaternary 2,3,8,9-tetraoxygenated benzo[*c*]phenanthridine alkaloids, which occur naturally in Papaveraceous and Rutaceous plants, have attracted much attention following the discovery of their potent antiviral [1] and anticancer activities [2] Fagaronine chloride **1** along with several other members of the benzo[*c*]phenanthridine family of alkaloids, has been reported to exhibit antileukemic activities in L-1210 and P-388 systems in mice [3]. Moreover, fagaronine has been shown to exhibit a cellular differentiation action on human leukemia K562 [4] and has recently been reported to inhibit topoisomerase I and II [5]. Fagaronine has been chosen as the reference compound for the assessment of HIV-RT inhibition by natural product extracts [6]. The closely related alkaloid nitidine **2** has been shown to inhibit AVM reverse transcriptase [7] and to stabilize the DNA-topoisomerase I complex [8].



1

fagaronine



2

nitidine

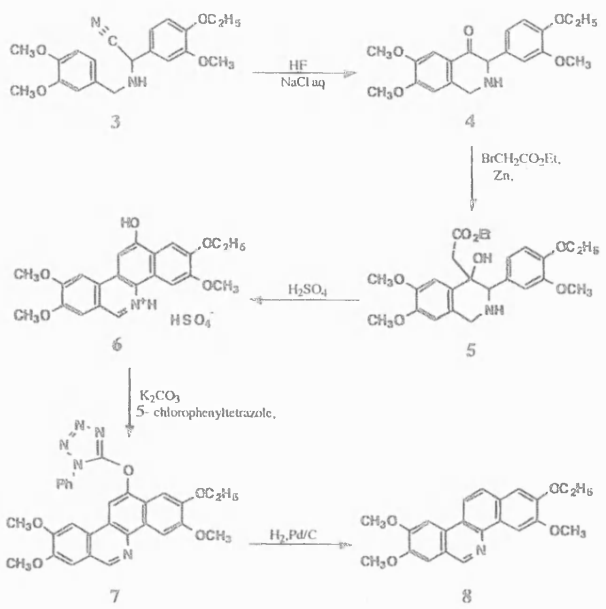
Although numerous synthetic and semi-synthetic methods may be found in the literature detailing the means of access of these alkaloids, relatively few efficient strategies are known for the synthesis of 2-hydroxy-3,8,9-trimethoxy-5-methylbenzo[*c*]phenanthridinium chloride, (fagaronine chloride). Existing syntheses [9] are long and employ low-yielding steps resulting in moderate overall returns. We have sought to overcome practical drawbacks in the preparation of benzo[*c*]phenanthridines and now report the first high-yielding total synthesis of fagaronine *via* a linear series of known chemical transformations amenable to scale-up procedures, employing relatively inexpensive reagents.

Results and discussion

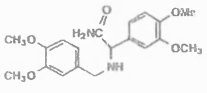
In this study, we succeeded in improving our existing method [10] for the synthesis of 2-ethoxy-3,8,9-trimethoxybenzo[*c*]phenanthridine (scheme 1), which allowed us to complete the crucial final steps in the synthesis of the natural product fagaronine.

The modified Strecker synthesis of the aminonitrile **3** was refined giving yields in excess of 95%. The reaction proceeded smoothly and cleanly when the rate of addition of the aqueous potassium cyanide solution to the imine was substantially reduced. Cyclization of the aminonitrile, conducted in anhydrous hydrogen fluoride, resulted in 95% yields of the aminoketone salt. The fluoro salt was found to be relatively soluble in water, which may account for previous inferior yields. Consequently, the crude product was stirred for 2 h in an aqueous saturated solution of sodium chloride until the counterion exchange was effected giving the insoluble hydrochloride salt of **4**. The solid was filtered and washed copiously with water giving the pure product as a white precipitate. During several concentration studies on a similar derivative, using decreasing quantities of HF in the cyclization, we isolated the intermediary primary amide **10**, whose structure was confirmed by ^{1}H and ^{13}C NMR.

* Correspondence and reprints

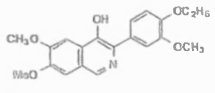


Scheme 1



10

Primary amide



11

Phenolic oxidation product

The Reformatski reaction, which introduces the two-carbon synthon required for the ensuing cyclization, was previously carried out using a very expensive high purity grade of zinc, giving a yield of 86%. We effected this reaction using zinc of modest purity with resulting yields of 65-70%. Whereas this reaction was conducted on the aminoketone free base, which is very prone to oxidation giving **11**, we have circumvented the need for this wasteful step (in which more than 10% of the product is lost) by carrying out the reaction directly on the dried salt. The cyclization of the analytically pure form of the β-hydroxyester **5** in sulfuric acid was complete in only 30 min with a yield of 70%. This short period of exposure most probably decreases sulfonation, and/or de-ethylation giving the water-soluble diphenol. The catalytic hydrogenation of the phenyltetrazole derivative **7** of the resulting phenolic sulfate **6** gave the deoxygenated 2-ethoxy-3,8,9-trimethoxybenzo[*c*]phenanthridine **8** in a yield of 70%. The product was found to adhere strongly to the Pd/C catalyst, which proved advantageous for the elimination of impurities by filtration. The pure compound was obtained thereafter from the continual extraction of the residual solid in chloroform using the Kumaguwa system.

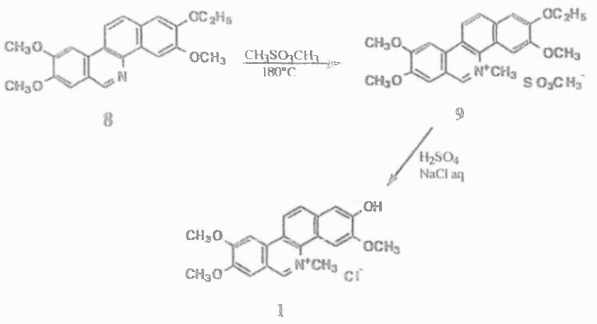
N-methylation at 170°C in the presence of methyl methanesulfonate proceeded very cleanly in a high yield. The *N*-methyl derivative **9** was subsequently treated with concentrated sulfuric acid to effect the lysis of the 2-ethoxy moiety (scheme 2).

An ^{1}H NMR study using 99% deuterated sulfuric acid (1% D_2O) showed that the optimum yield was obtained after 3 days. Fagaronine sulfate precipitated as a very fine suspension following the addition of the acidic reaction mixture to cold water. The product was adsorbed onto silica by the passage of the dilute acidic solution through a short column and was washed thoroughly with water. The product was liberated from the dried silica absorbent support by stirring in methanol. Following filtration, the solvent was removed under reduced pressure giving the crude fagaronine sulfate as a yellow/green solid. The trivial counterion exchange was effected by stirring the phenolic *N*-methyl sulfate in an aqueous solution of sodium chloride to yield the title compound **1**.

Conclusions

The overall yield for the synthesis of fagaronine from readily obtainable, inexpensive starting materials was 14%, a notable improvement over existing methods. This convenient strategy permits the isolation of large quantities of the pharmacologically active target alkaloid and will be of great value for the continual supply of fagaronine for biological tests and future studies directed towards the preparation of lipophilic derivatives. These species have already shown much promise in improving alkaloid bioavailability.

720



Scheme 2

Experimental section

General procedure

IR spectra were recorded on a Perkin-Elmer 640 spectrometer. IR absorption bands are quoted as weak (w), medium (m) or strong (s). NMR spectra were recorded in deuterated chloroform, unless otherwise stated, using a Jeol FT-NMR spectrometer operating at 270 MHz. Melting points were determined on a hot stage apparatus and repeated on an electrothermal melting point apparatus. All solutions were dried over anhydrous sodium sulfate before evaporation.

2-(3,4-Dimethoxybenzylamino)-2-(4-ethoxy-3-methoxyphenyl)acetonitrile **3**

Following a modified Strecker synthesis, 4-ethoxy-3-methoxybenzaldehyde (30.5 g, 169 mmol) in ethanol (340 mL) was added to an aqueous solution of 3,4-dimethoxybenzylamine (28.3 g, 169 mmol) made slightly acidic (pH 6) by the addition of dilute aqueous HCl. The resulting Schiff's base, formed *in situ*, was treated by the dropwise addition of an aqueous solution of potassium cyanide (16.7 g, 256 mmol/100 mL H_2O) over a period of several hours with vigorous stirring. Stirring was continued in this manner for 4 days during which the product precipitated as a light-yellow solid which was collected by filtration and thoroughly washed with water. The aminonitrile was dried under vacuum giving a yellow crystalline solid. (57.3 g, 95%), mp 59-60°C (lit 60-61°C), [10].

IR ν_{max} 3 320 (NH, m), 2 220 cm^{-1} (CN, w).

^{1}H NMR ($CDCl_3$) 1.46 (3H, t, J = 7 Hz, CH_3C), 3.44 (1H, d, J = 13 Hz, 4-CH_2), 3.77 (1H, d, J = 13 Hz, 4-CH_2); 3.87 (3H, s, OCH_3), 3.88 (3H, s, OCH_3), 3.89 (3H, s, OCH_3), 4.09 (2H, q, J = 7 Hz, O-CH_2), 4.81 (1H, s, CH), 6.93 (6H, m, Ar).

6,7-Dimethoxy-3-(4-ethoxy-3-methoxyphenyl)-1,2-dihydroisoquinolin-4(3H)-one (4) hydrochloride **4**

The aminonitrile (32 g, 89.9 mmol) was added portionwise to 60 mL of anhydrous hydrogen fluoride in a plastic bottle cooled externally with ice in a well-ventilated fume cupboard. The bottle was stoppered and the contents were stirred for 24 h. The bottle was opened and HF was allowed to evaporate over 4 h under a flow of nitrogen gas. The resulting brown viscous liquid was stirred with a saturated sodium chloride solution whereupon the hydrochloride precipitated as white solid with a brown hue (32.5 g, 95%). Purification of the analytical sample was effected by boiling with 96% ethanol which on cooling and filtering gave a white powder, mp 258-260°C; (lit 255-256°C) [10].

IR ν_{max} 1 680 cm^{-1} (CO, s).

^{1}H NMR ($CDCl_3$ + CF_3COH [1%]) 1.42 (3H, t, J = 7 Hz, CH_3C), 3.82 (3H, s, OCH_3), 3.98 (3H, s, OCH_3), 4.10 (2H, q, J = 7 Hz, CH_2C), 4.60 (2H, s, broad, CH_2), 5.41 (1H, s, broad, CH), 6.83 (3H, m, Ar), 6.97 (1H, s, Ar), 7.64 (1H, s, Ar), 8.60 (1H, s, broad, NH), 9.20 (1H, s, broad, NH).

Methyl-3-(4-ethoxy-3-methoxyphenyl)-hydroxy-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline-4-acetate **5**

All apparatus was thoroughly dried and left overnight in an oven at 100°C. Dioxan and dimethoxymethane were both refluxed with sodium metal for 1 h and distilled prior to use. The aminoketone salt was oven dried at 100°C for 3 days and stored under nitrogen immediately before the reaction.

To complete the characterization of this relatively insoluble compound, an analytical sample was liberated as its free base and was checked by spectroscopic methods.

IR ν_{max} 3 410 (NH, m), 1 685 cm^{-1} (CO, s).

^{1}H NMR ($CDCl_3$, free base) 1.43 (3H, t, J = 7 Hz, CH_3C), 2.28 (1H, s, broad, NH), 3.83 (3H, s, OCH_3), 3.91 (3H, s, OCH_3), 3.92 (3H, s, OCH_3), 4.05 (2H, q, J = 7 Hz, CH_2C), 4.10 (2H, s, CH_2–N), 4.51 (1H, s, CH-N), 6.61 (2H, s, Ar), 6.81 (1H, s, Ar), 6.94 (1H, s, Ar), 7.54 (1H, s, Ar).

The Reformatski reagent was prepared by the dropwise addition of ethyl bromoacetate (21 mL, 189 mmol) to a solution containing zinc, (13.5 g, 207 mmol, "mossy", 99% +) in dry dimethoxymethane, at such a rate as to maintain a slight reflux without external heating. Prior to the addition of the α-haloester, the zinc was activated by refluxing for 15 min with dimethoxymethane (45 mL) in the presence of trimethylsilyl chloride (2 mL). The solution darkened as the zinc was consumed and the characteristic green color of the organometallic complex appeared after a short period. Stirring was continued for 2 h after complete addition of the haloester.

Dioxan (30 mL) was added to the dry aminoketone salt (20 g, 51 mmol) to form a slurry to which the Reformatski reagent was added. The resulting solution developed a turbid brown color and gradually became homogeneous. Stirring was continued under nitrogen for 24 h at room temperature. Saturated aqueous ammonium chloride was added to the

solution and enough dichloromethane to form two layers. After filtration of residual suspended solids, the organic layer was removed and washed twice with ammonium chloride and once with water. The organic layer was dried over anhydrous sodium sulfate and the solvent was removed under reduced pressure to give a dark-brown residue. The residue was washed by swirling with a small portion of acetone to liberate the crude product as a light-brown solid following vacuum filtration (14.79 g, 65%). The pure product was obtained as a white powder following a second wash with a few drops of acetone, mp 182-184°C (lit 185-186°C) [10].

IR ν_{max} 3 400 (OH, m, broad), 3 300 (NH, m), 1 725 cm^{-1} (CO, s).

^1H NMR ($CDCl_3$) 1.09 (3H, t, J = 7 Hz, CH_3C, ester), 1.46 (3H, t, J = 7 Hz, CH_3C, ether), 2.68 and 3.08 (2H, AB system, J = 15 Hz, 4-CH_2), 3.80 (3H, s, OCH_3), 3.86 (3H, s OCH_3), 3.91 (3H, s, OCH_3), 2.95 (2H, q, J = 7 Hz, CH_2, ether), 4.07 (2H, q, J = 7 Hz, CH_2, ester), 3.90 (1H, d, J = 15 Hz, t-H), 4.17 (1H, d, J = 15 Hz, t-H), 4.86 (1H, s, 3-H), 6.54 (1H, s, Ar, 8-H), 6.83 (1H, d, J = 9 Hz, Ar), 7.02 (1H, s, 5-H), 7.06 (1H, d, J = 9 Hz, Ar), 7.37 (1H, s, Ar).

2-Ethoxy-3,8,9-trimethoxybenzo[c]phenanthridin-12-ol, sulfate 6

The tetrahydroisoquinolinol (4.5 g, 10.1 mmol) was added portionwise to concentrated sulfuric acid (25 mL, 98%), cooled externally by an ice bath, and stirred for 30 min. The resulting deep-red solution was added to cracked ice precipitating the yellow benzo[c]phenanthridinol sulfate (3.2 g, 66%) which was collected by filtration, mp > 320°C [10].

^1H NMR ($CDCl_3$, free base) 1.62 (3H, t, J = 7 Hz, CH_3C), 4.11 (3H, s, OCH_3), 4.22 (3H, s, OCH_3), 4.23 (3H, s, OCH_3), 4.32 (2H, q, J = 7 Hz, CH_2C), 7.41 (1H, s, Ar), 7.47 (1H, s, Ar), 7.91 (1H, s, Ar), 8.25 (1H, s, Ar), 8.82 (1H, s, Ar), 9.25 (1H, s, Ar).

2-Ethoxy-3,8,9-trimethoxy-12-(1-phenyl-1H-tetrazol-5-yloxy)benzo[c]phenanthridine 7

The benzo[c]phenanthridin-12-ol sulfate (3.2 g, 6.1 mmol) was added to dry acetone (50 mL) along with anhydrous sodium carbonate (3.5 g, 33.3 mmol). The solution was heated with stirring for 15 min until the free base had formed and the phenol dissolved. 5-Chloro-1-phenyl-1*H*-tetrazole (1.3 g, 7.3 mmol) was added to the mixture and the solution was stirred at reflux overnight. The solvent was removed under reduced pressure and the residual solid containing excess potassium carbonate was washed with dichloromethane to remove unreacted chlorophenyltetrazole. The product was obtained following the Soxhlet extraction of the chlorotetrazole-free solids from chloroform (8 h). The tetrazole derivative was recovered in pure form as a white solid (2.7 g, 77%), mp 259°C (lit 260-262°C) [10].

^1H NMR ($CDCl_3$) 1.49 (3H, t, J = 7.6 Hz, CH_3C), 4.05 (2H, q, J = 7.6 Hz, CCH_2), 4.10 (3H, s, OCH_3), 4.17 (3H, s, OCH_3), 4.19 (3H, s, OCH_3), 7.41 (1H, s, Ar), 7.62-7.80 (6H, m and s, Ar), 7.96 (1H, s, Ar), 7.99 (1H, s, Ar), 8.73 (1H, s, Ar), 8.76 (1H, s, Ar), 9.25 (1H, s, Ar).

2-Ethoxy-3,8,9-trimethoxybenzo[c]phenanthridine 8

The tetrazole derivative (1.5 g, 2.9 mmol) was partially dissolved in dioxan (70 mL) and further solubilized by ultrasound before being hydrogenated (H_2/9 atm) over palladium-on-charcoal (10%, 0.5 g) at 60°C for 24 h. The cooled mixture was filtered and the solid collected was subjected to continuous extraction in chloroform (7 h), by means of a Kumaguwa extractor, to give the benzo[c]phenanthridine (0.73 g, 70%), mp 269-272°C, (lit 271-274°C) [10].

^1H NMR ($CDCl_3$) 1.60 (3H, t, J = 7 Hz, CH_3C), 4.10 (3H, s, OCH_3), 4.16 (3H, s, OCH_3), 4.18 (3H, s, OCH_3), 4.30 (2H, q, J = 7 Hz, CCH_2), 7.28 (1H, s, Ar), 7.38 (1H, s, Ar), 7.83 (1H, d, J = 9 Hz, Ar), 7.88 (1H, s, Ar), 8.26 (1H, d, J = 9 Hz, Ar), 8.70 (1H, s, Ar), 9.27 (1H, s, 6-H).

2-Ethoxy-3,8,9-trimethoxy-5-methylbenzo[c]phenanthridine, methanesulfonate 9

The ethoxybenzo[c]phenanthridine (0.42 g, 1.2 mmol) was added to methyl methanesulfonate (4.5 mL, 53.1 mmol) in the presence of diisopropylethylamine (1.5 mL, 8.6 mmol) and refluxed on a metal alloy bath for 30 min at 170°C. Shortly after heating to the desired temperature, the solution became homogeneous and darkened in color. Upon cooling, acetone was added to the solution precipitating the product as a bright-yellow solid, which was filtered under vacuum and washed with dry ether (0.51 g, 93%), mp > 340°C dec, softens 223°C.

^1H NMR ($CDCl_3$ + CF_3CO_2H (1%)) 1.63 (3H, t, J = 7 Hz, CH_3C), 3.01 (3H, s, CH_3SO_3), 5.01 (3H, s, NCH_3), 4.12 (6H, s, 2×OCH_3), 4.27 (3H, s, OCH_3), 4.36 (2H, q, J = 7, CCH_2), 7.43 (1H, s, Ar), 7.74 (1H, s, Ar), 7.92 (1H, s, Ar), 8.00 (1H, s, Ar), 8.11 (1H, d, J = 9, Ar), 8.39 (1H, d, J = 9, Ar), 9.71 (1H, s, Ar).

MS (EI, 70 eV) m/z (%) 393 (M^+ + CH_3, 1.4%), 378 (M^+, 1.7%), 363 (M^+-CH_3, 100%), 334 (26%). Exact mass calculated for M^+-CH_3 $C_{22}H_{21}NO_4^+$: 363.147. Found : 363.146.

Fagaronine (2-hydroxy-3,8,9-trimethoxy-5-methylbenzo[c]phenanthridinium, chloride 1

The 2-ethoxy-5-methylbenzo[c]phenanthridine salt (300 mg, 0.63 mmol) was added to concentrated sulfuric acid (30 mL) and stirred at room temperature for 3 days under a nitrogen atmosphere. The resulting viscous, red-colored solution was added to cold water in the presence of ice to precipitate fagaronine sulfate as a fine yellow solid. The phenolic product was recovered by filtering the aqueous solution through silica gel and eluting the dried stationary phase with methanol. Removal of the solvent under reduced pressure gave the crude fagaronine sulfate as a yellow-green crystalline solid (198 mg, 70%).

^1H NMR ($CDCl_3$ + CF_3CO_2H [1%]) 4.13 (3H, s, OCH_3), 4.16 (3H, s, OCH_3), 4.30 (3H, s, OCH_3), 5.01 (3H, s, NCH_3), 7.62 (1H, s, Ar), 7.68 (1H, s, Ar), 7.98 (1H, s, Ar), 7.99 (1H, s, Ar), 8.12 (1H, d, J = 9 Hz, Ar), 8.41 (1H, d, J = 9 Hz, Ar), 9.43 (1H, s, Ar, 6-H).

MS (EI, 70 eV) m/z (%) 350 (M^+, 13%), 349 (M^+-H, 66.5%), 335 (M^+-CH_3, 100%). Exact mass calculated for $C_{21}H_{19}NO_4^+$ 349.1314. Found : 349.1317.

The counterion exchange was effected by stirring the sulfate in an 8% aqueous solution of sodium chloride [11]. The resulting bright-yellow precipitate was filtered and dried to give fagaronine chloride in an 88% yield, mp 198-200°C, (lit 202°C) [9].

^1H NMR (DMSO-d_6) 4.04 (3H, s, OCH_3), 4.11 (3H, s, OCH_3), 4.24 (3H, s, OCH_3), 5.11 (3H, s, NCH_3), 7.66 (1H, s, Ar), 7.94 (1H, s, Ar), 8.13 (1H, s, Ar), 8.16 (1H, d, J = 9 Hz, Ar), 8.63 (1H, s, Ar), 8.86 (1H, d, J = 9 Hz, Ar), 9.97 (1H, s, Ar).

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References

1 Suffness M, Cordell GA, *In : The Alkaloids*, Brossi A, Ed, Academic, New York, 1985, Vol 25, p 178
2 Cordell GA, Farnsworth NR, *Heterocycles* (1976) 4, 393
3 Messmer WM, Tin-Wa M, Fong HHS, Bevelle C, Farnsworth NR, Abraham DJ, Trojanek J, *J Pharm Sci* (1972) 61, 1858
4 Comoë L, Jeannesson P, Trentesaux C, Desoize B, Jardillier JC, *Leukemia Research* (1987) 5, 445
5 Larsen AK, Grondard L, Couprie J, Desoize B, Comoe L, Jardillier JC, Riou JF, *Biochem Pharmacol* (1993) 46, 8, 1403
6 Tan GT, Pezzuto JM, Kinghorn AD, Hugues SH, *J Nat Prod* (1991) 54, 143
7 Kakiuchi N, Hattori M, Namba T, Nishizawa M, Yamagishi T, Okuda T, *J Nat Prod* (1985) 48, 614
8 Fang SD, Wang LK, Hecht SM, *J Org Chem* (1993) 58, 19, 5025
9 Gillespie JP, Amoros LG, Stermitz FR, *J Org Chem* (1974) 39, 22, 3239
Ishii H, Chen IS, Ishikawa T, *Chem Pharm Bull* (1983) 31, 2963
Ishii H, Chen IS, Ishikawa T, *J Chem Soc Perkin Trans I* (1987) 671
10 Olugbade TA, Waigh RD, MacKay SP, *J Chem Soc Perkin Trans 1* (1990) 2657
11 Zee Cheng KY, Cheng CC, *J Heterocycl Chem* (1973) 10, 85

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Synthesis of β-amino esters using the Reformatsky reaction with α-amino nitriles

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Summary — The chemical behavior of α-amino nitriles towards the Reformatsky reagent has been studied. Under certain conditions, secondary aliphatic α-amino nitrile derivatives yielded a mixture of four products consisting of a β-amino ester, a β-lactam, a bulky tertiary amine and a cinnamic derivative. In an aliphatic and an alicyclic, fully nitrogen-substituted example, a β-aminoester is obtained in high yield as the sole product.

α-amino nitrile / β-amino ester / Reformatsky reaction

Résumé — Synthèse de β-aminoesters à partir de la réaction de Réformatsky sur les α-aminonitriles. Le comportement chimique des α-aminonitriles soumis aux conditions de la réaction Réformatsky a été étudié. Dans ces conditions, les α-aminonitriles tertiaires alicycliques ou aliphatiques conduisent aux β-aminoesters correspondants avec de bons rendements. Les α-aminonitriles secondaires conduisent à un mélange de β-aminoesters, un β-lactame, une amine tertiaire et un dérivé de l'acide cinnamique.

α-aminonitrile / β-aminoester / reaction Réformatsky

Introduction

In its classical form, the Reformatsky reaction involves the zinc-induced formation of β-hydroxyalkanoates from ethyl haloacetates and aldehydes or ketones [1]. α-Amino nitriles are extremely versatile synthons, easily obtained from the Strecker synthesis and frequently used in the synthesis of amino acids. We have recently exploited the virtues of such intermediates to construct substituted isoquinolinol derivatives, which upon further reaction led to the benzo[*c*]phenanthridine ring system [2a,b]. To the best of our knowledge, the Reformatsky-type reaction between α-bromo esters and α-amino nitriles has received no attention. Looking for organic intermediates containing more convenient functional groups for a straightforward access to our substituted structures, we herein describe the results of our studies using α-amino nitriles as carbanionic acceptor.

Results and discussion

We first considered the reaction with one of our intermediates **1a** under previously described reaction conditions [2]. The metal is activated using trimethylchlorosilane (TMCS) [3] and the reaction is conducted in the presence of ethyl bromoacetate in dimethoxymethane (DMM) at room temperature. The first attempts led to the isolation of mixtures of three products (scheme 1 and table I). Two main products were isolated: a β-amino ester **2a**, apparently resulting from a nucleophilic displacement of the cyano group of **1** and the β-lactam **3** resulting from an intramolecular nucleophilic attack. The third product, obtained in a low yield, was identified as the cinnamic ester derivative **4**.

The cyano group is known to react with the Reformatsky reagent to yield the corresponding β-keto ester [4]. In our reaction, which gave **2a**, no nucleophilic addition occurred on the cyano function. The formation of the β-aminoester **2a** is explained either by the direct nucleophilic displacement of the nitrile group borne on the α-carbon or the indirect nucleophilic addition on a transient imine. It has been reported that nitriles, in which the cyano group is weakly bonded to α-carbons, react with carbanionic species, such as the Grignard reagent, in a coupling process [4]. An intermediate nucleophilic imine addition has been proposed for the case of Grignard addition [4]. In a second step, an intramolecular nucleophilic reaction led to **3**. These two steps were only observed when the combination of trimethylchlorosilane (as zinc-activating reagent) and dimethoxymethane as solvent were used.

Similar results have previously been recorded using a nucleophilic Reformatsky-type attack on an

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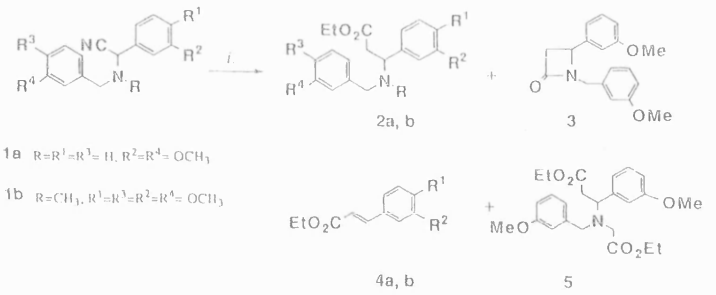


Scheme 1. *i*. Zn, $BrCH_2CO_2Et$, TMCS, DMM.

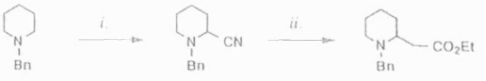
Table 1. Reaction of α-amino-nitriles **1a**, **1b** and **6** with the Reformatsky reagent (Zn, $BrCH_2CO_2Et$).

Substrate	*Solvent/metal activation*	*Zn/TMCS/$BrCH_2CO_2Et$ ratio*	*Yields (%)*[a]			
			2a,b/7	*3*	*4*	*5*
1a	DMM/TMCS	3.3:0.33:3.3	16	33	12	–
1a	DMM/TMCS	6.6:0.66:6.6	30	53	5	6
1a	Dioxane/ US/TMCS	6.6:0.66:6.6	46	15	3	–
1a	Dioxane/TMCS	6.6:0.66:6.6	17	5	3	35
1b	DMM/TMCS	3.3:0.33:3.3	87	–	–	–
6	DMM/TMCS	3.3:0.33:3.3	89	–	–	–

[a] Yields of isolated products isolated after chromatographic separation.

imine in the presence or not of a catalytic amount of trimethylchlorosilane in benzene or dimethoxymethane as solvents [5a,b]. In a recent work, additions of Reformatky reagents to imines in the presence of 1-(trimethylsilyl)benzotriazole afforded β-amino esters [5c]. Other authors have used amino trimethylsilyl derivatives as intermediates for the preparation of the monobactam antibiotic, thienamycin [6]. In our case, a possible transient silylated intermediate could enhance the reactivity of our secondary amine leading to the β-lactam structure. Attempts to improve this cyclisation led us first to use increasing amounts of organozinc and activator reagents (table 1). Besides the three compounds previously isolated an unexpected tertiary amine **5** was identified. Substituting the solvent, still in the presence of TMS, with a polar or less polar solvent such as tetrahydrofuran or benzene, was unfruitful. A striking improvement was observed when dioxane was used. Dioxane is known to be a poor solvent for the Reformatsky reaction except when ultrasonic (US) acceleration is associated [7]. Under these conditions our reaction failed. When TMCS was added, in conjunction with ultrasonic activation, the reaction led to an increase in the yield of **2a**. Without ultrasonic activation the main product isolated turned out to be the tertiary amine **5**. The presence of **4** may be explained through a retro-Michael reaction, with the loss of 3,4-dimethoxybenzylamine.

In order to extend the scope of this reaction, tertiary amines were studied as substrates. Under the usual conditions described above (table 1), **1b** yielded a sole product **2b**. Surprisingly, after purification of **2b** by column chromatography (silica gel, cyclohexane/ethyl acetate, 98:2 as eluent), a second product **4b**, corresponding to an elimination product, was isolated in very low yield, while with the identical reaction performed on the alicyclic compound **6** [8], only one product **7** was obtained in very high yield (scheme 2).



6 **7**

Scheme 2. *i*. $(PhIO)_n$, Me_3SiCN[8]; *ii*. Zn, $BrCH_2CO_2Et$, TMCS, DMM.

Conclusions

In conclusion, the Reformatsky reaction with a secondary aliphatic α-amino nitrile led to an unexpected mixture of two different amines, a β-lactam and a cinnamate derivative. Starting from tertiary amine analogues, this route represents a very facile access to functionalized amines when a β-amino ester synthon is required. Further investigations toward the application of this synthesis are currently in progress in our laboratory.

Experimental section

The ^1H and ^{13}C NMR spectra were recorded in deuterochloroform solutions (except when stated otherwise) on a JEOL GSX 270 WB spectrometer at 270.5 and 68 MHz respectively. Chemical shifts are reported in ppm relative to tetramethylsilane in deuterochloroform, except where otherwise specified. IR spectra were recorded on Perkin-Elmer 580 or 457 spectrophotometers using potassium bromide discs for solids, or neat liquid films for liquids. Only significant IR bands are quoted. Melting points were determined on a Electrothermal 8100 melting point apparatus and are uncorrected. HR mass spectra were determined on a high-resolution Varian MAT 311 or MS/MS ZabSpec TOF Micromass at 70 eV or using FAB.

General procedure

To a suspension of zinc dust (15.8 g) in dimethoxymethane (60 mL) under nitrogen, trimethylchlorosilane was added (2.80 mL, 0.024 mol) and the resulting mixture was stirred at room temperature for 15 min and then under reflux for 5 min. The suspension was cooled, and a solution of ethyl bromoacetate (27 mL, 0.24 mol) in dimethoxymethane (30 mL) is slowly added at such a rate that the solvent gently refluxes. After being heated to reflux for 2 h, the dark green solution is cooled and the corresponding amino nitrile **1** (0.073 mol) added. The mixture is then stirred for 6 h at room temperature. The mixture is poured over a solution of NH_4Cl (40 mL, saturated solution) and the organic layer is separated, eliminated under reduced pressure, and 30 mL of dichloromethane is added. The organic layer is washed three times with water (20 mL), dried (Na_2SO_4), and the solvent evaporated under reduced pressure to give an oily residue, which is then purified by column chromatography (cyclohexane/ethyl acetate: 70:30 to 95:5 and Merck 60H silica gel, 70–230 mesh).

◦ *Ethyl 3-[(3-methoxybenzyl)amino]-3-(3-methoxyphenyl)propanoate* **2a**

IR (KBr): ν(C=O) 1 720 cm^{-1}.

^1H NMR ($CDCl_3$, 270 MHz): 1.20 (t, CH_2–CH_3, 3H, J = 7.5 Hz); 2.25 (broad s, N*H*); 2.62 (dd, CH–C*H*$_2$–CO_2Et, 1H, J = 15.5 Hz, J = 9.0 Hz); 2.68 (dd, CH–C*H*$_2$–CO_2Et, 1H, J = 15.5 Hz, J = 5.00 Hz); 3.52 (d, Ar–C*H*$_2$–N, 1H, J = 13.5 Hz); 3.68 (d, Ar–C*H*$_2$–N, 1H, J = 13.5 Hz), 3.79, 3.81 (s, OCH_3, 6H); 4.1 (q, CH_2–CH_3, 2H, J = 7.5 Hz); 4.10 (dd, N–C*H*–Ar, 1H, J = 5.0 Hz, J = 9.0 Hz); 6.75–6.95 (m, *aromatic H*, 6H); 7.15–7.29 (m, *aromatic H*, 2H).

^{13}C NMR ($CDCl_3$, 68 MHz): 14.1 [OCH_2CH_3]; 42.9 [CH–CH_2]; 51.1 [Ar–CH_2–N]; 55.1, 55.2 [OCH_3]; 58.7 [N–CH–Ar]; 60.5 [O–CH_2–CH_3]; 112.4, 112.5, 112.9, 113.5, 119.4, 120.3, 129.2, 129.5 [*aromatic CH*]; 141.7, 144.0, 159.6, 159.8 [*aromatic C*]; 171.7 [C=O].

MS-IE: m/z (%): 343 (M^+, faible) 342 ($M-H^+$, 1), 256 (60), 136 (62), 121 (100).

HR-MS: calc for ($M-H^+$) 342.1705, found 342.1717.

◦ *Ethyl 3-[(3,4-dimethoxybenzyl)methylamino]-3-(3,4-dimethoxyphenyl)propanoate* **2b**

IR (KBr): ν(C=O) 1 720 cm^{-1}.

^1H NMR ($CDCl_3$, 270 MHz): 1.19 (t, CH_2–CH_3, 3H, J = 7.5 Hz); 2.12 (s, N–CH_3); 2.72 (dd, CH–CH_2–CO_2Et, 1H, J = 14.6 Hz, J = 7.5 Hz); 3.03 (dd, CH–CH_2–CO_2Et, 1H, J = 14.6 Hz, J = 8.0 Hz); 3.24 (d, Ar–C*H*$_2$–N, 1H, J = 13.0 Hz); 3.50 (d, Ar–C*H*$_2$–N, 1H, J = 13.0 Hz); 3.86, 3.88, 3.89, 3.90 (s, OCH_3, 12H); 4.10 (q, CH_2–CH_3, 2H, J = 7.5 Hz); 4.16 (dd, N–C*H*–Ar, 1H, J = 7.5 Hz, J = 8.0 Hz); 6.78–6.86 (m, *aromatic H*, 6H).

^{13}C NMR ($CDCl_3$, 68 MHz): 14.1 [OCH_2CH_3]; 37.6 [N–CH_3]; 37.9 [CH–CH_2]; 55.7 [OCH_3]; 55.8 [OCH_3]; 55.9 [2C, OCH_3]; 58.1 [Ar–CH_2–N]; 60.3 (O–CH_2–CH_3]; 63.9 [N–CH–Ar]; 110.5, 110.6, 111.7, 120.4, 120.6 [*aromatic CH*]; 130.9, 132.1, 147.9, 148.2, 148.5, 148.8 [*aromatic C*]; 171.9 [C=O].

HR-FAB (positive) MS: calc 418.223, found 418.223 ($M+H^+$).

◦ *1-(3-Methoxybenzyl)-4-(3-methoxyphenyl) azetidin-2-one* **3**

IR (KBr): ν(C=O) 1 750 cm^{-1}.

^1H NMR ($CDCl_3$, 270 MHz): 2.72 (dd, CO–C*H*$_2$–CH, 1H, J = 14.6 Hz, J = 2.2 Hz); 3.34 (dd, CO–C*H*$_2$–CH, 1H, J = 14.6 Hz, J = 5.2 Hz); 3.76, 3.78 (s, OCH_3, 6H); 3.78 (d, Ar–C*H*$_2$–N, 1H, J = 15 Hz); 4.39 (dd, N–C*H*–Ar, 1H, J = 5.2 Hz, J = 2.2 Hz); 4.75 (d, Ar–C*H*$_2$–N, 1H, J = 15 Hz); 6.60–6.80 (m, *aromatic H*, 6H); 7.20–7.10 (m, *aromatic H*, 2H).

^{13}C NMR ($CDCl_3$, 68 MHz): 42.1 [CO–CH_2–CH]; 46.7 [Ar–CH_2–N]; 53.5 [N–CH–Ar]; 55.1, 55.2 [OCH_3]; 111.7, 113.3, 113.8, 113.9, 118.7, 120.7, 129.7, 130.0 [*aromatic CH*]; 136.9, 139.5, 159.7, 159.9 [*aromatic C*]; 167.0 [C=O].

MS-IE: m/z (%): 297 (M^+, 31), 134 (100), 121 (26), 91 (22).

HR-MS: calc 297.1365, found 297.1369.

◦ *Ethyl trans-3-(3,4-dimethoxyphenyl)prop-2-enoate 4 [9]*

IR (KBr): ν(C=O) 1 685 cm^{-1}.

^1H NMR ($CDCl_3$, 270 MHz): 1.34 (t, CH_2–CH_3, 3H, J = 7.5 Hz); 3.83 (s, OCH_3, 6H); 4.26 (q, CH_2–CH_3, 2H, J = 7.5 Hz); 6.42 (d, CH=C*H*, 1H, J = 16 Hz); 6.93 (d, *H aromatic*, 1H, J = 8.5), 7.04 (d, *H aromatic*, 1H, J = 2.3 Hz); 7.12 (dd, *aromatic H*, 1H, J = 8.5 Hz, J = 2.3 Hz), 7.65 (d, C*H*=CH, 1H, J = 16 Hz).

^{13}C NMR ($CDCl_3$, 68 MHz): 14.3 [OCH_2–CH_3]; 55.9 [OCH_3]; 55.8 [OCH_3]; 46.7 [OCH_2–CH_3], 109.5, 110.9, 122.5 [*aromatic CH*]; 115.9, 144.5 [*CH*=], 127.3, 149.1, 151.0 [*aromatic C*]; 167.2 [*C*=O].

MS-IE: m/z (%): 236 (M^+, 65), 191 (100), 163 (27), 28 (26).

HR-MS: calc 236.1048, found 236.1048.

◦ *Diethyl 3-(3-methoxybenzyl)-4-(3-methoxyphenyl)-3-azahexanedioate* **5**

IR (KBr): ν(C=O) 1 720 cm^{-1} (broad).

^1H NMR ($CDCl_3$, 270 MHz): 1.15 (t, CH_2–CH_3, 3H, J = 7.3 Hz); 1.22 (t, CH_2–CH_3, 3H, J = 7.3 Hz); 2.71 (dd, CH–CH_2–CO_2Et, 1H, J = 14.5 Hz, J = 8.2 Hz); 3.00 (dd, CH–CH_2–CO_2Et, 1H, J = 14.5 Hz, J = 6.8 Hz); 3.16 (d, N–C*H*$_2$–CO_2Et, 1H, J = 17.2 Hz); 3.38 (d, N–CH_2–CO_2Et, 1H, J = 17.2 Hz), 3.59 (d, Ar–C*H*$_2$–N, 1H, J = 14.0 Hz); 3.79, 3.80 (s, OCH_3, 6H); 4.05 (q, CH_2–CH_3, 2H, J = 7.3 Hz); 4.10 (q, CH_2–CH_3, 2H, J = 7.3 Hz); 4.42 (dd, N–C*H*–Ar, 1H, J = 8.2 Hz, J = 6.8 Hz); 6.70–6.95 (m, *aromatic H*, 6H); 7.10–7.30 (m, *aromatic H*, 2H).

^{13}C NMR ($CDCl_3$, 68 MHz): 14.0 [OCH_2CH_3]; 14.2 [OCH_2CH_3]; 37.9 [CH–CH_2]; 51.2 [N–CH_2–CO_2Et]; 54.7 [Ar–CH_2–N]; 55.1, 55.2 [OCH_3]; 60.3, 60.4 [O–CH_2–CH_3]; 61.4 [N–CH–Ar]; 112.7, 112.9, 114.0, 114.0, 120.5, 120.9, 129.1, 129.2 [*aromatic CH*]; 140.7, 144.9, 159.5, 159.6 [*aromatic C*]; 171.3, 171.5 [*C*=O].

MS-IE: m/z (%): 384 (faible), 356 (44), 121 (100).

HR-FAB (positive) MS: calc 430.223, found 430.223.

• *1-Benzylpiperidine-2-carbonitrile* **6**

IR (KBr): ν(C≡N) 2 215 cm^{-1}.

^{1}H NMR ($CDCl_3$, 270 MHz): 1.55–1.95 (m, CH_2, 6H); 2.43 (m, CH_a–N, 1H); 2.80 (m, CH_b–N, 1H); 3.53 (d, Ar–CH_2–N, 1H, J = 13.2 Hz); 3.69 (d, Ar–CH_2–N, 1H, J = 13.2 Hz); 3.73 (t, CH–CN, 2H, J = 3.5 Hz); 7.25–7.35 (m, *aromatic H*, 5H).

^{13}C NMR ($CDCl_3$, 68 MHz): 20.4, 24.9, 28.5, 60.7 [alicyclic–CH_2]; 49.7 [Ar–CH_2–N]; 52.0 [CH–CN]; 116.7 [CN]; 127.5, 128.4, 128.9 [*aromatic CH*]; 136.9 [*aromatic C*].

• *Ethyl 1-Benzylpiperidine-2-acetate* **7**

IR (KBr): ν(C=O) 1 720 cm^{-1}.

^{1}H NMR ($CDCl_3$, 270 MHz): 1.24 (t, CH_2–CH_3, 3H, J = 7.3 Hz); 1.35–1.85 (m, CH_2, 6H); 2.18 (m, CH_2–N, 2H); 2.44 (dd, CH–CH_2–CO_2Et, 1H, J = 14.6 Hz, J = 8.0 Hz); 2.72 (dd, CH–CH_2–CO_2Et, 1H, J = 14.6 Hz, J = 5.0 Hz); 2.98 (m, CH–CH_2–CO_2Et, 1H); 3.37 (d, Ar–CH_2–N, 1H, J = 13.5 Hz); 3.82 (d, Ar–CH_2–N, 1H, J = 13.5 Hz); 4.13 (q, CH_2–CH_3, 2H, J = 7.3 Hz); 6.78–6.86 (m, *aromatic H*, 5H).

^{13}C NMR ($CDCl_3$, 68 MHz): 14.1 [OCH_2CH_3]; 22.2, 25.1, 30.8, 58.4 [alicyclic–CH_2]; 36.1 [CH–CH_2]; 50.6 [Ar–CH_2–N]; 57.5 [CH–CH_2]; 60.3 [O–CH_2–CH_3]; 126.7, 128.0, 128.4, 128.6 [*aromatic CH*]; 139.4 [*aromatic C*]; 172.8 [C=O].

MS-IE: m/z (%): 261 (M^+, weak), 174 (100), 91 (74).

HR-MS: calc 261.1729, found 261.1745.

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References

1 a) Fürstner A, *Synthesis* (1989), 571–590
b) Cintas P, In: *Activated Metals in Organic Synthesis*, CRC Press, Boca Raton, 1993

2 a) Lynch MA, Duval O, Pochet P, Waigh RD, *Bull Soc Chim Fr* (1994) 131, 718–722
b) Séraphin D, Lynch MA, Duval O, *Tetrahedron Lett* (1995) 36, 5731–5734

3 Picotin G, Miginiac P, *J Org Chem* (1987) 52, 4796–4798

4 Schaefer FC, In: *The Chemistry of the Nitrile Group* (Patai S, ed) John Wiley, London, 1970, 276–282

5 a) Palomo C, Cossio FP, Arrieta A, Odriozola JM, Oiarbide M, Ontoria JM, *J Org Chem* (1989) 54, 5736–5745
b) Dardoize F, Moreau JL, Gaudemar M, *Bull Soc Chim Fr* (1972), 3841–3846
c) Katritzky AR, Hong Q, Yang Z, *J Org Chem* (1995) 60, 3405–3408

6 Salzmann TN, Ratcliffe RW, Christensen BG, Bouffard FA, *J Am Chem Soc* (1980) 102, 6161–6163

7 Han BH, Boudjouk P, *J Org Chem* (1982) 47, 5030–5032

8 Moriarty RM, Vaid RK, Duncan MP, *Tetrahedron Lett* (1988) 29, 6913–6916

9 Pearl IA, Beyer DL, *J Org Chem* (1951) 16, 216–220

Table 5. *Selected geometric parameters (Å, °) for the title cations*

MAHMA		DMAHMA	
N1—C5	1.4816 (11)	N1—C5	1.4765 (9)
N1—H1N	0.901 (11)	N1—C6	1.4799 (9)
N1—H2N	0.903 (11)	N1—H1N	0.944 (9)
N1—H3N	0.943 (10)	N1—H2N	0.928 (9)
C5—N1	1.4815 (11)	C5—H5	0.953 (9)
C5—H5	0.930 (10)	C5—H6	0.971 (9)
C5—H6	0.939 (10)	C5—H7	0.984 (9)
C5—H7	0.987 (10)	C6—H8	0.990 (10)
		C6—H9	0.966 (9)
		C6—H10	0.960 (10)
C5—N1—H1N	108.7 (6)	C5—N1—C6	112.75 (6)
C5—N1—H2N	111.6 (7)	C5—N1—H1N	108.5 (5)
C5—N1—H3N	111.5 (6)	C5—N1—H2N	109.1 (5)
N1—C5—H5	106.8 (6)	N1—C5—H7	107.4 (5)
N1—C5—H6	109.3 (6)	N1—C6—H8	111.0 (5)
N1—C5—H7	108.7 (6)	N1—C6—H9	109.3 (5)
		N1—C6—H10	109.5 (6)

Table 6. *Selected geometric parameters (Å, °) for the maleate ions*

MAHMA		DAHMA	
C1—O1	1.2447 (8)	C1—O1	1.2396 (7)
C2—O1	1.2819 (9)	C1—O2	1.2915 (8)
C1—C2	1.4908 (10)	C1—C2	1.4911 (9)
C2—C2^{i}	1.3392 (14)	C2—C4	1.3359 (9)
C3—O3	1.2406 (9)	C4—C3	1.4920 (9)
C3—O4	1.2868 (9)	C3—O3	1.2381 (8)
C3—C4	1.4909 (10)	C3—O4	1.2874 (8)
C4—C4^{i}	1.3399 (14)		
O1—C1—C2—C2^{i}	167.57 (4)	O1—C1—C2—C4	178.87 (7)
O2—C1—C2—C2^{i}	−12.00 (9)	O2—C1—C2—C4	−1.65 (11)
O3—C3—C4—C4^{i}	177.39 (5)	C1—C2—C4—C3	−0.39 (13)
O4—C3—C4—C4^{i}	−2.36 (10)	C2—C4—C3—O3	−179.51 (7)
		C2—C4—C3—O4	0.29 (11)

Symmetry code: (i) $x, y, \frac{1}{2} - z$.

For both compounds, data collection: *CAD-4 EXPRESS* (Enraf–Nonius, 1992); cell refinement: *CAD-4 EXPRESS*; data reduction: *DREADD* (Blessing, 1987); program(s) used to solve structures: *SHELXS86* (Sheldrick, 1990); program(s) used to refine structures: *SHELXL93* (Sheldrick, 1993); molecular graphics: *ORTEPII* (Johnson, 1976).

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Supplementary data for this paper are available from the IUCr electronic archives (Reference: AB1534). Services for accessing these data are described at the back of the journal.

References

Blessing, R. H. (1987). *Cryst. Rev.* **1**, 1–58.

Enraf–Nonius (1992). *CAD-4 EXPRESS*. Version 1.1. Enraf–Nonius, Delft, The Netherlands.

Hamilton, W. C. (1965). *Acta Cryst.* **18**, 502–510.

Johnson, C. K. (1976). *ORTEPII*. Report ORNL-5138. Oak Ridge National Laboratory, Tennessee, USA.

Kalsbeek, N. & Larsen, S. (1991). *Acta Cryst.* C**47**, 1005–1009.

Larsen, S., Kozma, D. & Acs, M. (1994). *Acta Chem. Scand.* **48**, 32–36.

Olovsson, G., Olovsson, I. & Lehmann, M. S. (1984). *Acta Cryst.* C**40**, 1521–1526.

Popelier, P., Lenstra, A. T. H. & Geise, H. J. (1989). *Acta Cryst.* C**45**, 1024–1028.

Sequira, A., Rajagopal, H., Gupta, M. P., Vanhouteghem, F., Lenstra, A. T. H. & Geise, H. J. (1992). *Acta Cryst.* C**48**, 1192–1197.

Sheldrick, G. M. (1990). *Acta Cryst.* A**46**, 467–473.

Sheldrick, G. M. (1993). *SHELXL93. Program for the Refinement of Crystal Structures*. University of Göttingen, Germany.

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2,8-Dimethylphenoxathiin 10-Oxide

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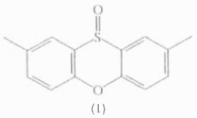
Abstract

An important precursor to biologically active compounds, 2,8-dimethylphenoxathiin 10-oxide ($C_{14}H_{12}O_2S$), is found to adopt a folded geometry. The dihedral angles between the aromatic rings are 11.8 (2) and 15.4 (2)° for the two independent molecules, with the S atoms lying out of the ring planes.

Comment

The synthesis of phenoxathiins is of current interest because they show a variety of biological activities. Some have antibacterial properties (Gavriliu *et al.*, 1996; Maior *et al.*, 1995), others have exhibited antitumor activity (Palmer *et al.*, 1988), and yet others can be used as antidepressants (Cooper *et al.*, 1992). These compounds may also be used as catalysts, for example, in chlorination reactions (Mais & Fiege, 1990) and in hydroformulation reactions (Kranenburg *et al.*, 1995).

The molecular structures of the two crystallographically independent conformations of the title compound, (I), are shown in Fig. 1. They are approximately related by a non-crystallographic inversion centre at (0.56,0.26,0.38). Both display a folded geometry with dihedral angles between the least-squares planes of the aromatic rings of 11.8 (2) and 15.4 (2)°. Thus, the molecule is flatter than the related unsubstituted



(I)

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compound phenoxathiin 10-oxide (PTO), where the corresponding angle is 28° (Chen *et al.*, 1979). This is not, however, a simple fold about the S···O axis of the heterocyclic ring, for although the O atoms are coplanar with the aromatic rings the S atoms lie out of the plane. Examination of the geometric parameters (Table 1) reveals that the bond lengths are in good agreement with those in PTO, but that the internal bond angles of the heterocyclic ring are slightly larger in (I). This is consistent with a flattened geometry [C7—S1—C1 96.76 (16), C21—S2—C15 95.98 (16), C6—O2—C12 120.8 (3) and C20—O4—C26 120.3 (3)°, compared with 94.8 (3) and 118.8 (4)° in PTO]. The sulfoxide group lies nearly normal to the ring systems, as is shown by the torsion angles O1—S1—C1—C6 and O3—S2—C15—C16 [92.1 (3) and 86.5 (3)°, respectively; see also Table 1].

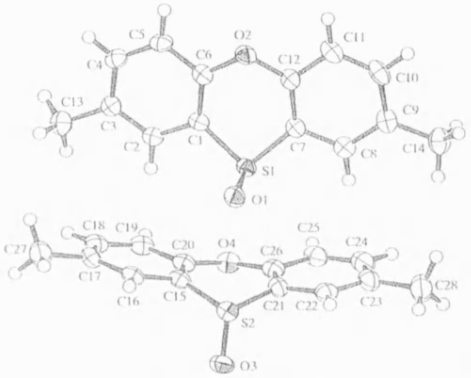

Fig. 1. The molecular structure of (I) with 50% probability ellipsoids and H atoms as small spheres of arbitrary size.

Experimental

2,8-Dimethylphenoxathiin (5.2 g, 22.8 mmol) was heated under reflux overnight in the presence of ethanol (125 ml) and hydrogen peroxide (15 ml, 27%). An additional amount of hydrogen peroxide (10 ml, 27%) was added and the solution heated for a further 3 h. The reaction mixture was cooled to room temperature, then the volume of solvent was reduced by half *in vacuo*. The white solid obtained was dissolved in toluene and purified by silica-gel column chromatography. The first fraction, assigned as 2,8-dimethylphenoxathiin 10,10-dioxide was obtained in 8% yield. Ethyl acetate was then used to elute (I) in 75% yield (m.p. 403–405 K). Solutions of (I) left in air were found to oxidize slowly back to 2,8-dimethylphenoxathiin 10,10-dioxide.

Crystal data

$C_{14}H_{12}O_2S$
M_r = 244.30
Mo $K\alpha$ radiation
λ = 0.71069 Å
Monoclinic
$P2_1/c$
a = 13.274 (2) Å
b = 12.214 (4) Å
c = 15.044 (3) Å
β = 110.606 (14)°
V = 2282.9 (10) Å^3
Z = 8
D_x = 1.422 Mg m^{-3}
D_m not measured
Cell parameters from 24 reflections
θ = 17.0–19.6°
μ = 0.268 mm^{-1}
T = 123 K
Cut needle
0.40 × 0.40 × 0.35 mm
Colourless

Data collection

Rigaku AFC-7S diffractometer
$\omega/2\theta$ scans
Absorption correction: none
4676 measured reflections
4481 independent reflections
2980 reflections with $I > 2\sigma(I)$
R_{int} = 0.038
θ_{max} = 26.01°
$h = 0 \rightarrow 16$
$k = 0 \rightarrow 15$
$l = -18 \rightarrow 17$
3 standard reflections
every 150 reflections
intensity decay: 4.85%

Refinement

Refinement on F^2
$R[F^2 > 2\sigma(F^2)]$ = 0.058
$wR(F^2)$ = 0.170
S = 1.036
4481 reflections
311 parameters
H atoms riding
$w = 1/[\sigma^2(F_o^2) + (0.0831P)^2 + 1.7748P]$ where $P = (F_o^2 + 2F_c^2)/3$
$(\Delta/\sigma)_{max} < 0.001$
$\Delta\rho_{max}$ = 0.337 e Å^{-3}
$\Delta\rho_{min}$ = −0.659 e Å^{-3}
Extinction correction: none
Scattering factors from *International Tables for Crystallography* (Vol. C)

Table 1. *Selected geometric parameters (Å, °)*

S1—O1	1.499 (2)	S2—C21	1.772 (3)
S1—C7	1.766 (3)	O2—C6	1.376 (4)
S1—C1	1.775 (4)	O2—C12	1.381 (4)
S2—O3	1.503 (3)	O4—C20	1.370 (4)
S2—C15	1.768 (4)	O4—C26	1.388 (4)
O1—S1—C7	107.38 (15)	O3—S2—C21	107.75 (15)
O1—S1—C1	107.46 (15)	C15—S2—C21	95.98 (16)
C7—S1—C1	96.76 (16)	C6—O2—C12	120.8 (3)
O3—S2—C15	106.70 (15)	C20—O4—C26	120.3 (3)
O1—S1—C1—C6	92.1 (3)	O3—S2—C15—C20	−84.9 (3)
O1—S1—C1—C2	−84.3 (3)	O3—S2—C15—C16	86.5 (3)
S1—C1—C6—O2	7.7 (5)	S2—C15—C20—O4	−11.4 (5)
O1—S1—C7—C12	−93.5 (3)	O3—S2—C21—C26	86.2 (3)
O1—S1—C7—C8	83.4 (3)	O3—S2—C21—C22	−86.9 (3)
S1—C7—C12—O2	−5.0 (5)	S2—C21—C26—O4	6.8 (5)

H atoms were refined as riding, including free torsion of the methyl groups. U_{iso} values for H atoms on aromatic C atoms were set to 1.2 times the U_{eq} values of the parent atoms and to 1.5 times the U_{eq} values for those in methyl groups.

Data collection: *MSC/AFC Diffractometer Control Software* (Molecular Structure Corporation, 1985). Cell refinement: *MSC/AFC Diffractometer Control Software*. Data reduction: *TEXSAN* (Molecular Structure Corporation, 1993). Program(s) used to solve structure: *SIR* (Burla *et al.*, 1989). Program(s) used to refine structure: *SHELXL*97 (Sheldrick, 1997). Molecular graphics: *ORTEP*II (Johnson, 1976). Software used to prepare material for publication: *SHELXL*97.

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References

Burla, M. C., Camalli, M., Cascarano, G., Giacovazzo, C., Polidori, G., Spagna, R. & Viterbo, D. (1989). *J. Appl. Cryst.* **22**, 389–393.
Chen, J. S., Watson, W. H., Austin, D. & Ternay, A. L. (1979). *J. Org. Chem.* **44**, 1989–1991.
Cooper, B. R., White, H. L., Beck, O., Norton, R. M., Rigdon, G. C., Howard, J. L., Kraemer, G. W. & Ferris, R. M. (1992). *Drug Dev. Res.* **25**, 181–190.
Gavriliu, D., Stoia, S. & Maior, O. (1996). *Rev. Chem. (Bucharest)*, **47**, 326–331.
Johnson, C. K. (1976). *ORTEPII*. Report ORNL-5138. Oak Ridge National Laboratory, Tennessee, USA.
Kranenburg, M., van der Burgt, Y. E. M., Kamer, P. C. J., van Leeuwen, P. W. N. M., Goubitz, K. & Fraanje, J. (1995). *Organometallics*, **14**, 3081–3089.
Maior, O., Gavriliu, D. & Stoia, S. (1995). *S. Braz. J. Chem.* **3**, 79–88.
Mais, F. J. & Fiege, H. (1990). European Patent No. 0 399 293.
Molecular Structure Corporation (1985). *MSC/AFC Diffractometer Control Software*. MSC, 3200 Research Forest Drive, The Woodlands, TX 77381, USA.
Molecular Structure Corporation (1993). *TEXSAN. Single Crystal Structure Analysis Software*. Version 1.6. MSC, 3200 Research Forest Drive, The Woodlands, TX 77381, USA.
Palmer, B. D., Rewcastle, G. W., Atwell, G. J., Baguley, B. C. & Denny, W. A. (1988). *J. Med. Chem.* **31**, 707–712.
Sheldrick, G. M. (1997). *SHELXL97. Program for the Refinement of Crystal Structures*. University of Göttingen, Germany.

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2-(Imidazol-1-yl)-1-(2-naphthyl)ethanone Oxime

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Abstract

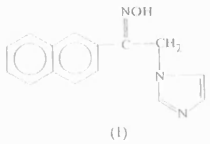
In the title compound, imidazol-1-ylmethyl 2-naphthyl ketone oxime, $C_{15}H_{13}N_3O$, the naphthalene and imidazole rings are essentially planar. The oxime group is twisted by 36.2 (1)° out of the naphthalene plane. The oxime configuration is *Z*. The structure is stabilized by intra- and intermolecular hydrogen bonds.

Comment

Oximes show geometric isomerism due to the double bond between the N and C atoms. The reaction of hydroxylamine hydrochloride with an unsymmetrical ketone may result in either a mixture of two isomeric oximes or only one of the isomers, depending on the structure of the ketone or the reaction conditions (Mixich & Thiele, 1979; Migrdichian, 1957). Because of the great differences in physical, chemical and biological properties of the geometric isomers, determination of the configuration of the isomers is important (Mathison *et al.*, 1989).

Oximes and oxime ethers have a broad pharmacological activity spectrum, encompassing antifungal, antibacterial, antidepressant and insecticidal activities, as well as activity as a nerve-gas antidote, depending on the pharmacophoric group of the molecule (Polak, 1982; Balsamo *et al.*, 1990; Holan *et al.*, 1984; Forman, 1964). An oximino group usually modifies the activity or sometimes is directly responsible for the activity.

In connection with our interest in the anticonvulsant compound nafimidone and antifungal–antibacterial agents with (arylalkyl)azole structures, we have prepared nafimidone oxime (Walker *et al.*, 1981). Since the structure of this oxime is important with respect to the activity and configuration of the O-ether derivatives of this compound that have been prepared in our laboratory, we studied its spectral properties and molecular geometry by UV, IR, ^{1}H NMR, mass spectroscopy, elemental analysis and X-ray crystallography. We report here the structure of nafimidone oxime, (I).



(I)

The naphthalene moiety is essentially planar, with bond lengths and angles in good agreement with those observed in other naphthalene derivatives (Elmali *et al.*, 1995; Irngartinger *et al.*, 1993). The imidazole ring is also planar [$\Sigma(\Delta/\sigma)^2 = 1.6$]. The dihedral angle between these two planes is 96.98 (8)°. Some significant differences are observed for the bond distances in the imidazole ring compared with the averages derived from the Cambridge Structural Database quoted by Allen *et al.* (1987) [given in square brackets]: N1—C13 1.360 (3) [1.349 (18)], N1—C15 1.335 (2) [1.370 (10)], N2—C14 1.364 (3) [1.376 (11)], N2—C15 1.305 (3) [1.313 (11)] and C13—C14 1.344 (3) Å [1.360 (14) Å]. In two other imidazole oxime derivatives, all the C—N bond distances in the imidazole ring are intermediate between the expected single- and double-bond lengths (Grassi *et al.*, 1993; Bruno *et al.*, 1994). The exocyclic angles around the N1 atom show considerable asymmetry. However,

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Neuromuscular blocking agents. The preparation and properties of *cis*- and *trans*- D- and L-norcoralydine methiodides

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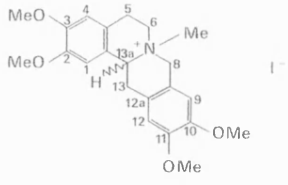
Cis- and *trans*-isomers of D- and L-norcoralydine methiodide have been separated chromatographically and their neuromuscular blocking potencies measured against tubocurarine on the chick biventer cervicis muscle preparation.
Conformations have been assigned to the four isomers on the basis of their NMR and circular dichroism spectra, and NMR spectra of the corresponding ethiodides. The relative potencies of the four norcoralydine methiodides and the related laudanosine methiodides have been explained in terms of a favoured tetrahydroisoquinolinium conformation for interaction with the postulated anionic receptor.

Most series of quaternary ammonium neuromuscular blocking agents show falling potencies, becoming less depolarising and increasingly curare-like, as quaternary methyl groups are successively replaced by other larger N-alkyl substituents (1). These changes in pharmacological response reflect both electronic and steric effects. The former spread the charge density at the quaternary centre across adjacent α-carbon atoms (2, 3, 4), influencing the strength of the electrostatic interaction with the anionic binding centre(s), whilst steric effects determine the closeness of fit of the quaternary groups at the effective binding site (5, 6, 7).

The marked influence on potency of steric effects due to chain-branching close to the quaternary centre has been demonstrated in flexible molecules such as D-, L- DL- and *meso*-isomers of succinyl di-α- and β-methylcholine di-iodides (8, 9, 10). Thus, equipotent molar ratios relative to suxamethonium (1) for succinyl di-β-methylcholine di-iodides were L-(+)-, 887, D-(−)-, 1 200 and DL-(*meso*)-, 913.

The norcoralydine methiodides (I) are structurally and stereochemically related to the tetrahydropapaverinium sub-units of tubocurarine and related bisbenzylisoquinolinium alkaloids. Their more rigid structures provide an unequivocal method of distinguishing steric from electronic effects in the action of quaternary compounds at the neuromuscular junction. The present paper describes the separation, pharmacological evaluation and stereochemical assignments of *cis*- and *trans*- D- and L-norcoralydine methiodides. The *trans*-isomers have rigid *trans*-decalin-like conformations, but the *cis*-isomers are flexible and may adopt one of two alternative *cis*-decalin-like conformations. Evidence is presented in favour of one of two possible *cis*-conformations.



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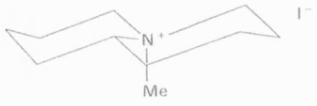

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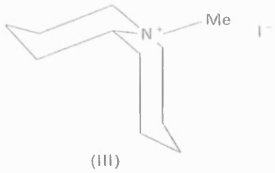


(III)

CHEMISTRY

MOYNEHAN, SCHOFIELD, JONES and KATRITZKY (11, 12) have shown by infrared and ^1H-NMR spectroscopy that the ring system in quinolizidine exists predominantly in

the *trans*-fused conformation, and that quinolizidine methiodide, prepared by direct methylation of quinolizidine, similarly has the *trans*-fused conformation with the N-methyl group orientated axially (II). Quinolizidine methiodide prepared by ring-closure leads on the other hand mainly to the *cis*-compound. In our experience, however, direct methylation of quinolizidine gives a product which on the evidence of its NMR spectrum contains some *cis*-isomer (III).

Similarly, treatment of D- and L-norcoralydines with methyl iodide gave methiodides, each of which showed evidence of the presence of two isomers by their NMR spectra and by thin-layer chromatography on cellulose with 0.1N hydrochloric acid (R_F 0.35 and 0.86), with the isomer mixtures containing some 25-30 % of the minor component (R_F 0.86). Column chromatography of each methiodide in 0.1N hydrochloric acid on cellulose afforded separations to give pure *cis*- and *trans*- D- and L-norcoralydine methiodides.

The isomers show characteristic differences in their ultraviolet, infrared, circular dichroism and ^1H-NMR spectra. The minor isomer has been assigned the *cis*-fused configuration and the major isomer the *trans*-fused configuration on the basis of their 100 MHz NMR spectra. The two isomers showed marked differences in their solubilities. The *cis*-racemate was much more soluble in $CDCl_3$ than the *trans*-racemate. The optically active forms of the *trans*-isomer, however, were significantly more soluble than the racemate, sufficient for spectroscopic examination.

According to MOYNEHAN, SCHOFIELD, JONES and KATRITZKY (12), the N-methyl groups absorb at lower field in *cis*-fused N-methylquinolizidinium ions (τ 6.85) than in the *trans*-fused isomers (τ 7,04). 100 MHz NMR spectra of the norcoralydine methiodides showed in the major product an intense $\overset{+}{N}-CH_3$ signal at τ 6.92 (axial with respect to rings B and C), with a corresponding signal at τ 6.41 (equatorial with respect to ring B, axial with respect to ring C, or *vice versa*) in the minor isomer, in accord with the tentative assignment of configuration as the *trans*- and *cis*-fused isomers respectively. The latter is in good agreement with the signal at τ 6,48 (equatorial $\overset{+}{N}-CH_3$) in D-laudanosine hydrochloride. None-the-less, we have sought and obtained additional evidence of these assignments elsewhere in the spectrum.

BHACCA, CYMERMAN-CRAIG, MANSKE, ROY, SHAMMA and SLUSARCHYK (13) have shown that the 100 MHz spectrum of cularine (IV) shows a pair of doublets at τ 5.56 attributable to the C-1 proton which is spin-coupled in an ABX pattern (J_{AX} = 4 and J_{BX} = 12 Hz) to the two non-identical protons at C-9, in agreement with the conformation (IV). By analogy, if the hydrogen at C-13a is designated as H_X and the two hydrogens at C-13 are designated H_A and H_B, NEWMAN projections of *trans*-norcoralydine methiodide (V) and the two possible conformations of *cis*-norcoralydine methiodide (VI and VII) show that whereas H_A and H_B are equivalent (both dihedral angles *ca* 60° to H_X) in the *cis*-isomer of conformation (VI), they are non-equivalent with dihedral angles of *ca* 60° (AX) and *ca* 180° (BX) respectively in the *trans*-isomer and the *cis*-isomer of conformation (VII).

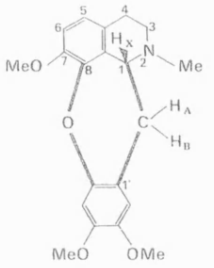
The signal patterns for the 13a protons are complicated by the additional signals arising from the two C-8 protons which lie in a closely similar environment, and appear in the spectrum of the *trans*-isomer in $CDCl_3$ only as part of a complex pattern of three protons intensity in the τ 4,60-5.00 region. These signals, however, are clearly separated in DMSO appearing as a pair of doublets (1 proton) at τ 4.90 (J_{AX} = 4, J_{BX} = 12 Hz) attributable to the C-13a proton, and two doublets (1 proton each) at τ 5.16 and τ 5.39 showing geminal coupling (J = 14 Hz) due to the two protons at C-8. The corresponding signals in the *cis*-isomer are somewhat more clearly resolved in $CDCl_3$ than those of the *trans*-isomer, and also appear as a pair of doublets of one proton intensity (C-13a) at τ 4.70 (J_{AX} = 4, J_{BX} = 12 Hz) and a singlet (two C-8 protons) at τ 4.88.

The identity of the two isomers and the assignment of conformation (VII) to the *cis*-isomer is confirmed by the C-13 proton signals, which in the latter isomer fall within the shielding cone of ring A. As a result, the C-13 proton signals are at much lower field in the *trans*-isomer and appear as a pair of multiplets, each of 1 proton intensity at τ 5.25 and τ 5.50. In contrast, comparable signals are not evident in the *cis*-isomer, since, as shown by the integral, they form part of the complex pattern of 9 protons (7 in the *trans*-isomer) in the region τ 6.2-7.2, which apart from the $\overset{+}{N}-CH_3$ signals is poorly resolved in both isomers.

We have similarly prepared and separated the *cis*- and *trans*-isomers of D-norcoralydine ethiodide. As anticipated, the isomers are identified by their NMR spectra which show characteristic $\overset{+}{N}.CH_2.CH_3$ triplets at τ 8,9 (J = 7) in the major product, and at τ 8.43 (J = 8) in the minor product. The signal at higher field in the major product is wholly consistent with its assignment as the *trans*-isomer, since the $\overset{+}{N}.CH_2.CH_3$ group falls within the shielding cones of both aromatic rings. Likewise, the lower field signal in the minor product accords with the *cis*-conformation (as VII), since the $\overset{+}{N}.CH_2.CH_3$ group falls within the shielding cone of only one (ring A) of two aromatic rings.

The C-13a proton is seen in the *trans*-ethiodide as a pair of doublets centred at τ 4.30 (J_{AX} = 5, J_{BX} = 13 Hz), clearly separated from the pair of doublets due to the two C-8 protons at τ 4.70 and 5.08 showing geminal coupling (J = 17). In contrast the C-13a proton signal is shifted marginally upfield in the *cis*-isomer due to the $\overset{+}{N}.CH_2.CH_3$ group, and shows only as part of a poorly resolved complex of three protons intensity in the region τ 4.7 to 5.1 attributable to both C-13a and C-8 protons. The *trans*-ethiodide also shows a pair of multiplets (each 1 proton) at τ 5.60 and 5.78 attributable to the two C-13 protons, which are not apparent in the *cis*-isomer since, as in the *cis*-methiodide, they form part of the complex pattern in the τ 6.2-7.0 region.

The ultraviolet and circular dichroism spectra of *cis*- and *trans*-(+)-norcoralydine methiodide also show small, but characteristic differences which reflect the differences of molecular shape. The ultraviolet absorption of the *cis*-isomer shows an intensification of the benzenoid local



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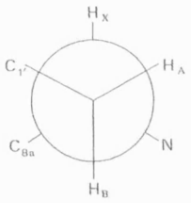

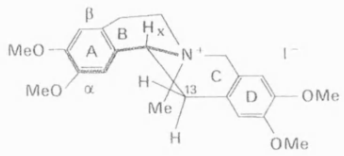

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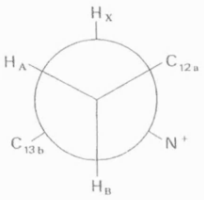

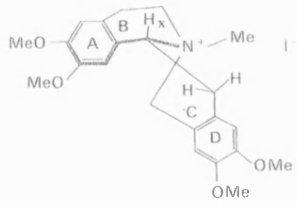

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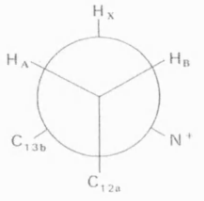

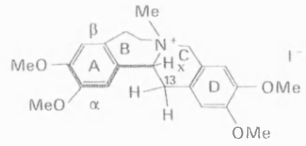

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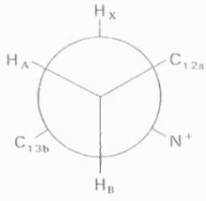


excitation band at 286 nm compared with the *trans*-isomer, and also shows a small maximum at 350 nm, which is not evident in the *trans*-isomer. These spectral differences are reflected in the circular dichroism spectra of *cis*- and *trans*-D-norcoralydine methiodides. Thus, whereas the *trans*-isomer shows only two positive maxima at 210 and 240 nm respectively, the *cis*-isomer shows an additional band which appears as a negative minimum at 278 nm in correspondance with the long-wavelength ultraviolet absorption maximum (fig. 1).

PHARMACOLOGICAL RESULTS AND DISCUSSION

As anticipated, all the compounds show a low level of potency compared with that of (+)-tubocurarine. Nonethe-less, potency differences (table 1) between the four isomeric norcoralydine methiodides are highly significant ($P < 0.01$). In contrast to the related laudanosine methiodides, of which the L-compound is more potent than the

TABLE 1

Relative molar neuromuscular blocking potencies

Compound	Relative molar potency (TC = 100) ± S.E.M.
D-(+)-*cis*-Norcoralydine methiodide	13.6 ± 0.75
D-(+)-*trans*-Norcoralydine methiodide	4.3 ± 0.15
L-(−)-*cis*-Norcoralydine methiodide	2.1 ± 0.06
L-(−)-*trans*-Norcoralydine methiodide	3.2 ± 0.15
L-(+)-Laudanosine methiodide	8.1 ± 0.75
D-(−)-Laudanosine methiodide	2.2 ± 0.15

D-isomer (14), both *cis*- and *trans*-D-norcoralydine methiodides are more potent than the corresponding L-isomers. Significantly, the circular dichroism spectra of both *cis*- and *trans*-norcoralydine methiodides (fig. 1) also show positive maxima at 210 and 240 nm, corresponding to that of L-laudanosine methiodide (15). Comparison of these CD spectra is valid, since the laudanosines and norcoralydines have identical methoxyl substitution patterns (16, 17). Correspondance of the CD spectra of both D-norcoralydine isomers at these wavelengths with that of L-laudanosine

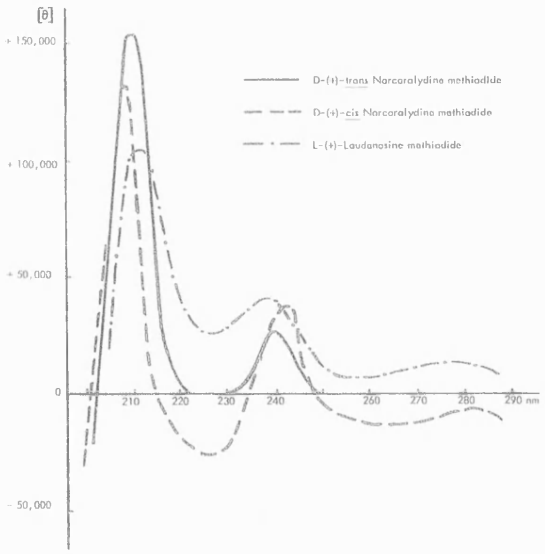


Fig. 1. — Circular dichroism curves of

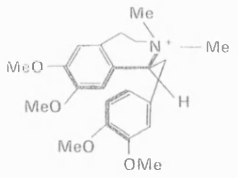
D-(+)-*trans*-Norcoralydine methiodide.
D-(+)-*cis*-Norcoralydine methiodide.
L-(+)-laudanosine methiodide.

methiodide reflects their general chiral similarity, comcomprising a combination of both 2nd (ring B) and 3rd chiral spheres (rings C and D), rather than that of the second chiral spheres alone (16, 17, 18).

None-the-less, it is perhaps significant that of the two norcoralydine methiodides, only the D-*cis*-isomer shows a CD spectrum corresponding in all three maxima at 210, 240 and 280 nm with that of L-laudanosine. This not only accords with the fact that the conformation of the tetrahydroisoquinolinium moiety comprising rings A and B in the more potent D-*cis*-isomer is identical with that of the conformation of L-laudanosine methiodide (VIII) (14, 19, 20, 21), but also strongly suggests the very close overall molecular shape of these two molecules in solution. Similar considerations determine the relative potency of the four isomers.

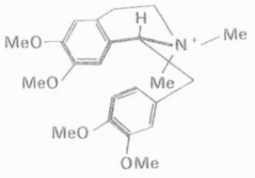
Since D-*cis*- and *trans*-norcoralydine methiodides have different A/B ring conformations, but identical C/D ring conformations, and since the A/B ring conformation of the D-*cis*-isomer is also identical with that of the more potent L-laudanosine methiodide, it appears that the A/B tetrahydroisoquinolinium conformation plays a key rôle in determining the potency of such compounds. Furthermore, the markedly angular shape of D-*cis*-norcoralydine methiodide is such that rings C and D represent a major hindrance to receptor interaction at the α-face of the molecule. Consequently, onium ion-receptor interaction at the unhindered upper β-face of the A/B tetrahydroisoquinolinium moiety is strongly favoured.

The β-face of the less potent D-*trans*-norcoralydine methiodide is similarly unhindered, but this face presents an A/B tetrahydroisoquinolinium group of opposite conformation to that of the D-*cis*-isomer and identical with that in D-laudanosine methiodide (IX). On the other hand, the α-face of the D-*trans*-isomer is such that either ring B or ring C (but not the entire tetrahydroisoquinolinium moieties) presents a similar, but not identical, conformational aspect to a potential anionic receptor as the β-face of ring B in the D-*cis*-isomer. Rings C and D of the D-*trans*-isomer, however, lie in a plane at an angle of about 30° to that of rings A and B. Rings C and D, therefore, project below the plane of the molecule, so that it would be hindered in any such alignment of the α-face to an anionic receptor. Hence, the lower potency of the D-*trans*-isomer.



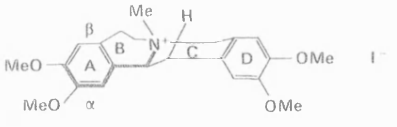
(VIII)

The concept of a sterically favoured receptor interaction in neuromuscular block is supported by the low potency of L-*trans*-norcoralydine methiodide (X). This is capable of presenting its β-face to an anionic receptor in the same favourable β-face orientated conformation as the D-*cis*-



(IX)

isomer. The β-face approach, however, is subject to steric hindrance by rings C and D which project upwards in a plane angled at about 30°. Additionally, two axial hydrogens protrude prominently from the β-face of the L-*trans*-compound at C-5 and C-13, both in 1,3-locations with respect to the onium N-methyl group, compared with only one such at C-8 in the more active D-*cis*-compound.



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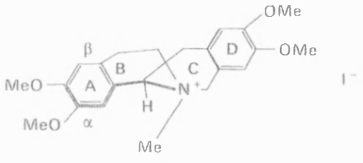

(XI)

Onium group receptor interactions ar the β-face of the least active isomer, L-*cis*-norcoralydine methiodide (XI), are even less favoured due to the combination of the unfavourable A/B ring conformation and strong steric hindrance from rings C and D, which are angled upwards at about 60° on the β-face.

EXPERIMENTAL

Melting points are uncorrected. Analytical results for C, H, N and I were obtained within experimental error of the theoretical value, and are not reported in the paper. NMR spectra were determined in deuterochloroform with TMS as internal standard on a Perkin-Elmer R14 by Dr. P. Bladon of this University, to whom we are indebted for this service. Circular dichroism spectra were recorded in methanol on a Cary, Model 6001, recording spectropolarimeter fitted with a circular dichroism attachment.

L-(−)-*Norcoralydine Hydrochloride*

L-(−)-Norcoralydine hydrochloride was prepared from L-(+)-tetrahydropapaverine hydrochloride as described by Corrodi and Hardegger (22) as colourless needles (from methanol), m.p. 204-206° (decomp.), $[\alpha]_D^{20}$ − 242.2° (c, 0.785 EtOH). Lit. m.p. 225-230°, $[\alpha]_D$ − 227° (c, 1.0 EtOH).

L-(−)-*Norcoralydine*

L-(−)-Norcoralydine was prepared from L-(−)-norcoralydine hydrochloride, as colourless needles (from methanol), m.p. 185-186°, $[\alpha]_{D2}^{22.5}$ − 298.4° (c, 0.892, $CHCl_3$), R_F 0.80 in $CHCl_3$ – EtOH (90 : 10) on thin-layer silica gel G (Merck). Corrodi and Hardegger (22) report m.p. 177°, $[\alpha]_D$ − 277° (c, 1.0, $CHCl_3$). *Anal.* for $C_{21}H_{25}O_4N$: C, H, N.

cis- and *trans*-L-(−)-*Norcoralydine Methiodides*

L-(−)-Norcoralydine (2.02 g) was refluxed with methyl iodide (15 ml) for 3.5 hr. Excess methyl iodide was removed by evaporation, the product washed with dry ether and dried over P_2O_5 under vacuum. Thin-layer chromatography of the crude product in 0.1N hydrochloric acid on Whatman Cellulose Chromedia CC41 showed two components R_F 0.35 and 0.86. The crude product (2.02 g) was chromatographed on a column (40 × 3.5 cm) of Whatman Cellulose Chromedia CC31-celite (50 : 50) in 0.1N hydrochloric acid.

The minor component (thin-layer R_F 0.86), *cis*-L-(−)-*norcoralydine methiodide* was obtained from methanol-ether as a yellowish solid (0.044 g), m.p. 150-151°, $[\alpha]_D^{20}$ − 167.2° (c, 0.204 in MeOH). *Anal.* for $C_{22}H_{28}NO_4I,H_2O$: C, H, N.

The major component (thin-layer R_F 0.35) trans-L-(−)-*norcoralydine methiodide* was obtained from methanol as a pale yellow solid (0.174 g), m.p. 247-249°, $[\alpha]_D^{20.8}$ − 132.8° (c, 0.308 in MeOH). *Anal.* for $C_{22}H_{28}NO_4I,H_2O$: C, H, N.

D-(+)-*Norcoralydine Hydrochloride*

D-(+)-Norcoralydine hydrochloride was prepared from D-(−)-tetrahydropapaverine hydrochloride (3 g) by the method of Corrodi and Hardegger (22). D-(+)-*Norcoralydine hydrochloride* was obtained as colourless needles (3.1 g) from methanol, m.p. 203-205° (decomp.), $[\alpha]_D^{20}$ + 241.6° (c, 0.896, EtOH). *Anal.* for $C_{21}H_{26}NO_4Cl$: N.

D-(+)-*Norcoralydine*

D-(+)-Norcoralydine was prepared as for the L-isomer, and obtained as colourless crystals from methanol, m.p. 185-186°, $[\alpha]_D^{22}$ + 298.8° (c, 1.239 in $CHCl_3$). R_F 0.80 in $CHCl_3$ – EtOH (90 : 10) on thin-layer silica gel G (Merck). *Anal.* for $C_{21}H_{25}O_4N$: C, H, N.

cis- and *trans*-D-(+)-*Norcoralydine Methiodides*

cis- and *trans*-D(+)-Norcoralydine methiodides were prepared (2.8 g) and separated as described for the L-isomers. The minor component (thin-layer R_F 0.86), *cis*-D-(+)-*norcoralydine methiodide* was obtained from methanol-ether as a yellowish solid (0.078 g), m.p. 150-152°, $[\alpha]_D^{19.8}$ + 165.8° (c, 0.405 in MeOH). Circular dichroism (c, 0.02052 in MeOH) $[\theta]_{282}$ − 6,000, $[\theta]_{265}$ − 13,300, $[\theta]_{242.5}$ + 38,900, $[\theta]_{230}$ − 25,400 $[\theta]_{207.5}$ + 133,000. *Anal.* for $C_{22}H_{28}NO_4I,H_2O$: C, H, N.

The major component (thin-layer R_F 0.35), trans-D-(+)-*norcoralydine methiodide*, was obtained from methanol as a pale yellow solid (0.320 g), m.p. 248-250°, $[\alpha]_D^{19.8}$ + 134.5° (c, 0.430 in MeOH). Circular dichroism (c, 0.02126 in MeOH) $[\theta]_{240}$ + 28,000, $[\theta]_{227}O$, $[\theta]_{210}$ + 154,500. *Anal.* for $C_{22}H_{28}NO_4I,H_2O$: C, H, N.

cis- and *trans*-D-(+)-*Norcoralydine Ethiodides*

cis- and *trans*-D-(+)-Norcoralydine ethiodides were prepared and separated as described for the corresponding methiodides. The minor component (thin-layer R_F 0.85), cis-D-(+)-*norcoralydine ethiodide* was obtained from methanol, m.p. 113.5-116.5°, $[\alpha]_D^{21}$ + 143.2° (c, 0.221 in MeOH). *Anal.* for $C_{23}H_{30}NO_4I,H_2O$: C, H, N.

The major component (thin-layer R_F 0.40), trans-D-(+)-*norcoralydine ethiodide*, was obtained from methanol, m.p. 180-182°, $[\alpha]_D^{20.5}$ + 136.7° (c, 0.201 in MeOH). *Anal.* for $C_{23}H_{30}NO_4I,H_2O$: C, H, N.

L-(+)-*Laudanosine Methiodide*

Circular dichroism (c, 0.01946 in MeOH), $[\theta]_{280}$ + 13,500, $[\theta]_{257}$ + 6,800, $[\theta]_{237.5}$ + 41,500, $[\theta]_{226}$ + 26,000, $[\theta]_{211}$ + 105,500.

Neuromuscular blocking potency

The compounds were tested on the isolated biventer cervicis muscle from chicks (23) as described in the accompanying paper (14).

Because of their water insolubility, the isomeric D- and L-norcoralydinium iodides were initially dissolved in dimethylsulphoxide (DMSO) and subsequently diluted with Krebs-Henseleit solution. Large concentrations of DMSO (30-50 mg/ml) themselves modify neuromuscular transmission in the biventer cervicis muscle preparation (24), possessing both facilitatory and blocking properties. Lower concentrations of DMSO (8-20 mg/ml) do not themselves modify neuromuscular transmission, but slightly modify the blocking action of tubocurarine. In order to reduce the influence of the DMSO in the experiments described in this paper, the maximum final bath concentration of DMSO used was 8 mg/ml, and the norcoralydinium compounds were always compared with tubocurarine solutions containing an identical quantity of DMSO. All the compounds tested produced a non-depolarizing type of neuromuscular blockade, exhibiting no propensity to cause contracture of muscle, and reducing the response to exogenous acetylcholine (30 µg/ml).

Résumé

Les isomères *cis*- et *trans*- des formes D et L de l'iodométhylate de la norcoralydine ont été séparés par chromatographie et leurs pouvoirs curarisants ont été évalués par rapport à la tubocurarine sur une préparation de muscle « biventer cervicis » de poussin.

On a déterminé les conformations des quatre isomères d'après leurs spectres de RMN et de dichroïsme circulaire, et les spectres RMN des iodoéthylates correspondants.

Les pouvoirs respectifs des quatre iodométhylates des norcoralydine et laudanosine associées ont été explicités à la faveur d'une conformation tétrahydroisoquinolinium lors de l'interaction avec le récepteur anionique.

Zusammenfassung

Cis- und *Trans*isomere des D- and L-Norcoralydin Methiodid sind chromatographisch getrennt worden, wobei ihre neuromuskulären Blockierkräfte dem Tubocararin gegenüber bei der Praparation des Muskels *biventer cervicis* des Kükens abgemessen worden ist. Verschiedene Gestaltungen sind den vier Isomeren aufgrund der NMR und kreisförmigen Dichroismenspektren wie auch die NMR-Spektren der entsprechenden Ethiodide zugeschrieben worden.

Die relativen kräfte der vier Norcoralydin Methiodide und der verwandten Laudanosinen Methiodiden läsz sich erklären, mit Rucksicht auf die Gestaltung mit einem Tetrahydroisochinolinium der am Häufigsten mit dem vorausgesetzten anionschen Rezeptoren zusammenwirkt.

Bibliography

(1) J. B. Stenlake, *Progress in Medicinal Chemistry*, editors Ellis, G. P. and West, G.B., London : Butterworths, 1963, **3**, 1.
(2) P. E. B. Holmes, D. J. Jenden and D. B. Taylor, *Nature* (London), 1947, **159**, 86.
(3) D. B. Taylor, *Pharmac. Rev.*, 1951, **3**, 412.
(4) J. Thomas and G. A. Starmer, *J. Pharm. Pharmacol.*, 1961, **13**, 752.
(5) E. J. Ariens and W. M. De Groot, *Arch. internation. Pharmacodyn. Thérap.*, 1954, **99**, 193.
(6) S. Thesleff and K. R. Unna, *J. Pharmacol. exper. Thérapeut.*, 1954, **111**, 99.
(7) F. M. Carey, D. Edwards, J. J. Lewis and J. B. Stenlake, *J. Pharm. Pharmacol.*, 1959, **11**, 701.
(8) A. H. Beckett, N. J. Harper and J. W. Clitherow, *J. Pharm. Pharmacol.*, 1963, **15**, 349.
(9) E. Lesser, *J. Pharm. Pharmacol.*, 1961, **13**, 703.
(10) E. Lesser, *J. Pharm. Pharmacol.*, 1966, **18**, 408.
(11) T. M. Moynehan, K. Schofield, R. A. Y. Jones and A. R. Katritzky, *Proc. chera. Soc.*, 1961, 218.
(12) T. M. Moynehan, K. Schofield, R. A. Y. Jones and A. R. Katritzky, *J. chem. Soc.*, 1962, 2637.
(13) N. S. Bhacca, J. Cymerman-Craig, R. H. F. Manske, S. K. Roy, M. Shamma and W. A. Slusarchyk, *Tetrahedron*, 1966, **22**, 1467.
(14) J. B. Stenlake, W. D. Williams, N. C. Dhar and I. C. Marshall, *Chim. Ther.*, 1974, **9** (3), 233.
(15) J. Cymerman-Craig, M. Martin-Smith, S. K. Roy and J. B. Stenlake, *Tetrahedron*, 1966, **22**, 1335.
(16) G. Snatzke, M. Kajtar and F. Werner-Zamojska, *Advanced Institute on ORD and CD*, Tirrenia (Pisa), Italy, Sept 5-18, 1971, 26.
(17) G. Snatzke, J. Hrbek, L. Hruban, A. Horeau and F. Santavy, *Tetrahedron*, 1970, **26**, 5013.
(18) G. Snatzke and P. C. Ho, *Tetrahedron*, 1971, **27**, 3645.
(19) I. G. Marshall, J. B. Murray, G. A. Small and J. B. Stenlake, *J. Pharmacol.*, 1967, **19**, Suppl. 53S.
(20) J. B. Stenlake, *Ann. pharm. fr.*, 1968, **26**, 185.
(21) J. Fridrichsons and A. Mc L. Mathieson, *Tetrahedron*, 1968, **24**, 5785.
(22) N. Corrodi and E. Hardegger, *Helv. chim. Acta*, 1956, **39**, 889.
(23) B. L. Ginsborg and J. Warriner, *Brit. J. Pharmacol. Chemotherapy*, 1960, **15**, 410.
(24) I. G. Marshall and A. Gandhia, *J. Pharm. Pharmacol.*, 1972, **24**, 417.

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Biodegradable neuromuscular blocking agents. I. Quaternary esters

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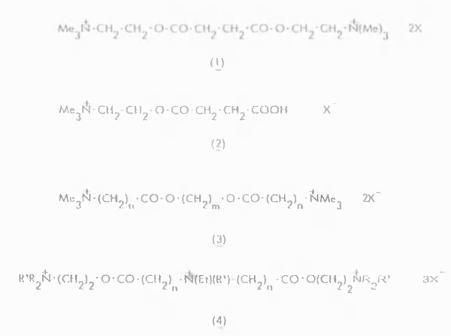
A new approach to the preparation of metabolically-degradable, short-acting neuromuscular blocking agents, modelled on the facile Hofmann degradation of the quaternary alkaloid petaline, **5**, has been examined. A series of 1-ethoxycarbonylmethyltetrahydroisoquinolinium and polymethylene-*bis*-1-ethoxycarbonylmethyltetrahydroisoquinolinium compounds has been prepared. The compounds had low neuromuscular blocking potencies, and underwent only partial Hofmann elimination *in vitro* at physiological pH. The elimination products underwent a retro-Hofmann reaction, and equilibria with the quaternary salt were slowly attained.

To date biodegradation and short duration of action in neuromuscular blocking agents has been achieved almost exclusively by incorporation of ester groups, which are subject to enzymic hydrolysis and hence fragmentation of the molecule. Thus Suxamethonium, **1**, owes its short action to hydrolysis by non-specific plasma esterases (1, 2, 3) into succinylmonocholine, **2**, and choline both of which have negligible neuromuscular blocking potency (4, 5). Suxamethonium is widely used clinically for muscle relaxation, but suffers from a number of important disadvantages. Thus, it induces neuromuscular block by depolarisation of the motor end plate, and in consequence block is not readily reversed by anticholinesterases such as Neostigmine and Edrophonium. Depolarisation also raises plasma potassium, a phenomenon which can lead to cardiac arrest in patients with hyperkalaemia (6). Suxamethonium also induces fasciculation prior to onset of paralysis, which results in painful post-operative cramps (7), and repeated doses can give rise to asystole due to vagal stimulation (8). A further serious hazard which attends the use of Suxamethonium is that its action may be unduly prolonged resulting in severe apnoea in patients with a deficiency of plasma esterases due to liver disease or to genetically determined deficiencies (9, 10, 11).

A number of other related ester-linked quaternary compounds have been examined as potential short-acting neuromuscular blocking agents, including the reversed



(1)

(2)

(3)

(4)

esters (12), **3** and the tris-onium compounds (13), **4** (R = Me or Et ; n = 1 or 2). The former suffer from the same disadvantages as Suxamethonium, whilst the latter proved insufficiently potent to warrant development for clinical use. All other muscle relaxants in general use, including Tubocurarine chloride, Gallamine triethiodide, Alcuronium bromide (14, 15), Pancuronium bromide (16, 17, 18), Fazadinium (AH 8165 ; 19), although competitive non-depolarising neuromuscular blocking agents and readily reversible by anticholinesterases, are slower in onset and significantly longer acting than Suxamethonium in man. There still remains, therefore, a need for a really short-acting non-depolarising neuromuscular blocking

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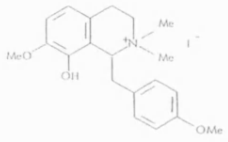
agent for use in intubation and surgical procedures. Such an agent should also desirably be both non-depolarising and free from the vagal-stimulating effects shown by all the major competitive blockers currently in use. The latter result in tachycardia, and arterial hypertension, which is the cause of excessive bleeding during surgery (20).

The general structural similarity of the simple mono-quaternary ammonium alkaloid, petaline (21), **5**, to the benzylisoquinolinium units of tubocurarine, and the ease with which it undergoes Hofmann elimination in solution on Amberlite IRA 400 (OH) ion exchange resin to yield petalinemethine, **6**, suggested the possibility of exploiting similar eliminations effective at physiological pH (7.4) as an alternative and novel means of terminating neuromuscular blocking action in a new approach to short-acting muscle relaxants.



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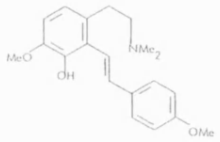


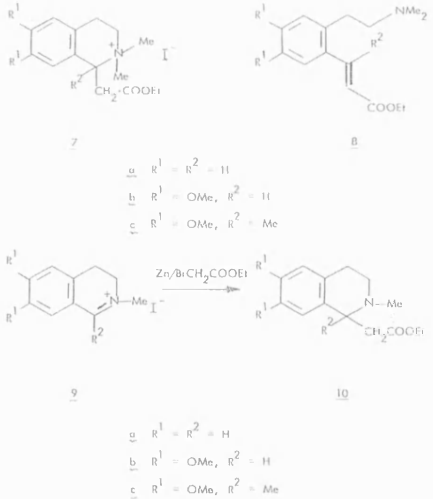
6

CHEMISTRY

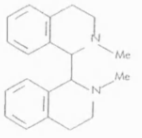
In an attempt to delineate the essential parameters for continuing rapid Hofmann elimination at physiological pH with an effective level of competitive neuromuscular block, we have confined our attention initially to a number of simple isoquinolinium compounds. The β-amino ester methiodide (22), **7b**, which undergoes an almost instantaneous Hofmann elimination to the unsaturated ester, **8b**, in aqueous sodium hydroxide formed the basis of the present study.

The ester, **7b**, is readily available by Bishler-Napieralski cyclisation of ethyl *N*-3,4-dimethoxyphenethylmalamate followed by reduction and methylation of the product (22, 23). However, both this route (24) and the Pictet-Spengler synthesis (25) seemed insufficiently flexible for synthesis of the particular 1-substituted-1,2,3,4-tetrahydroisoquinolines envisaged in this work. Accordingly, we turned our attention to the alternative nucleophilic addition of Grignard and Reformatski reagents to 3,4-dihydroisoquinolinium salts. Similar nucleophilic additions to isoquinolinium salts are known (26, 27) but this route to 1-substituted-1,2,3,4-tetrahydroisoquinoline has not found favour as a general method because of the instability of the intermediate 1-substituted-1,2-dihydroisoquinolines. Nucleophilic addition to 3,4-dihydroisoquinolines, however, is free from this disadvantage, and further, offers the advantage that the required 1-substituted products are obtainable from preformed dihydroisoquinolines of known constitution.

The esters, **7a** and **b**, were prepared from the corresponding 3,4-dihydroisoquinolinium salts, **9a** and **b** (Scheme 1) by a Reformatski type condensation with zinc and ethyl bromoacetate (28) and methylation of the product. The only difficulty encountered in the Reformatski addition arose from the poor solubility of the 3,4-dihydroisoquinolinium salt in the usual reaction solvents. Tetrahydrofuran gave the cleanest products, but it was necessary to add the quaternary salt in solid form. Simultaneous introduction of the solid and bromoester to the zinc suspension proved difficult, and the most satisfactory procedure was found to be portionwise addition of the salt either prior to or after addition of the bromoester. In the preparation of **10a**, substantial amounts of the by-product, **11**, were obtained. This is formed presumably by a bimolecular reduction of the 3,4-dihydroisoquinolinium iodide in a reaction analogous to the reduction of 3,4-dihydro-1-methylisoquinolines with aluminium amalgam (29, 30).



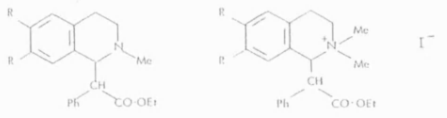
Scheme 1



11

The facility with which the quaternary salts, **7a** and **b**, undergo Hofmann elimination must be due largely to the effect of the ester carbonyl in increasing the acidity of the adjacent methylene hydrogen atoms. Introduction of a phenyl or second alkoxy carbonyl group at this position

should still further increase the acidity of the remaining hydrogen atom. The esters, **12a**, **12b**, **14a** and **14b**, were prepared by addition of sodio ethyl phenylacetate and the sodio malonate esters respectively to the appropriate 3,4-dihydroisoquinolinium salts (**9**). The phenylmalonate ester, **12a**, and its desdimethoxy analogue, **12b**, were readily methylated to stable methiodides, **13a** and **13b**.

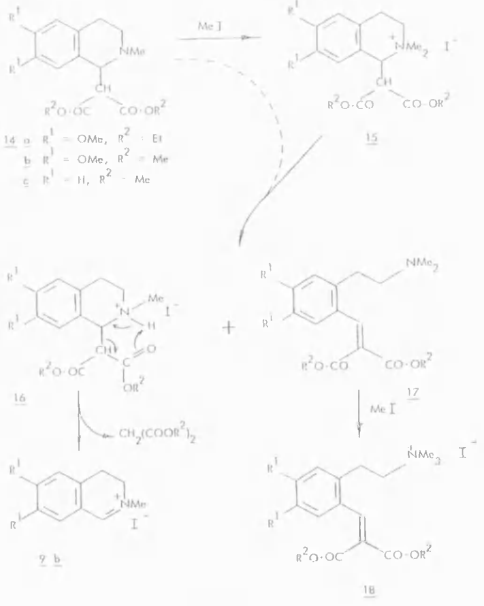


12 a R = MeO
b R = H

13 a R = MeO
b R = H

In contrast, the methiodide, **15**, of the malonate ester, **14a**, proved to be unstable and could not be isolated. Instead, reaction of **14a** with methyl iodide gave a mixture of the 3,4-dihydroisoquinolinium iodide, **9b**, the quaternary salt, **18**, and diethyl malonate via **16** and **17**. Analysis of the PMR spectrum of the products formed in $CDCl_3$ showed the reaction to have the following stoichiometry :

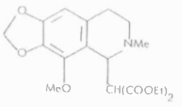
2 **14a** + 2 MeI ⟶ **9b** + **18** + $CH_2(COOEt)_2$



14 a R^1 = OMe, R^2 = Et
b R^1 = OMe, R^2 = Me
c R^1 = H, R^2 = Me

Scheme 2

The corresponding dimethyl ester, **14b**, and the desdimethoxydimethyl ester, **14c**, behaved similarly with methyl iodide. The stoichiometry and products are explained by the reaction sequence (Scheme 2). LIEBERMANN and GLAWE (31) obtained analogous products from the base, **19**, and methyl iodide, which gave cotarnine iodide, **20**, and the quaternary benzylidenemalonic ester, **21**. The base, **19**, is also readily cleaved in cold dilute hydrochloric acid to cotarnine chloride and diethylmalonate.



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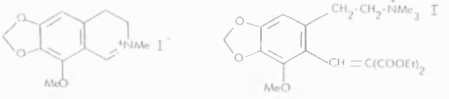


20 21

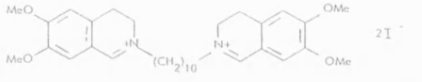
Since the monoquaternary salts were expected to possess only a low level of neuromuscular blocking activity, the decamethylene-bis-quaternary salt, **24**, was prepared by treatment of the decamethylenebis-3,4-dihydroisoquinolinium compound, **22**, with ethyl bromoacetate and zinc, and methylation of the reaction product, **23**. As described below, Hofmann elimination of the bisquaternary compound, **24**, proceeded slowly and only to the extent of some 20 % at pH 7.4. In order to circumvent the possibility that elimination is unnecessarily retarded by concomitant hydrolysis of the ethoxycarbonyl to the corresponding, less powerfully electron-attracting carboxyl groups, we have also synthesised the bisquaternary compound, **25**, by condensation of **9b**, with ethyleneglycol bisphenylacetate, **26**, in the presence of sodamide in two stages, followed by methylation of the resulting ditertiary base, **27**. Enzymic ester hydrolysis of this compound, whilst similarly unmasking free carboxylic acid groups capable of retarding Hofmann elimination, would at the same time give rise to two monoquaternary compounds with attendant low neuromuscular blocking activity.

HOFMANN ELIMINATION STUDIES

Hofmann elimination of the quaternary esters proceeded almost instantaneously on addition of a slight excess of dilute NaOH solution to aqueous solutions of the salts. Our finding that their neuromuscular blocking action.

although low, as expected, was not of short duration prompted us to examine in detail the behaviour at pH 7.4 of the monoquaternary salt, **7b**, and the hydriodide of its Hofmann elimination product, the cinnamic ester, **8b**. The acids, **28** and **29**, which are the hydrolysis products of **7b** and **8b**, respectively, were also examined to investigate the possibility that ester hydrolysis at pH 7.4 might be confusing the picture.

The behaviour of compounds, **7b** and **8b**, at pH 7.4 was followed by comparison of changes in their UV spectra with time (Figs. 1 and 2) with corresponding spectra of the two compounds in water. Hofmann elimination is characterised by the disappearance of the 283 nm band and the appearance of two stronger bands at 292 nm and 329 nm. Figures 1 and 2 show that both the quaternary salt, **7b**, and the cinnamic ester, **8b**, slowly reach equilibrium. The small changes in the spectrum of **7b** (Fig. 1), which contrast with the very much larger changes in the spectrum of the corresponding cinnamic ester, **8b**, are consistent with equilibrium concentrations equivalent to about 80 % quaternary salt, **7b**, and 20 % cinnamic ester, **8b**.



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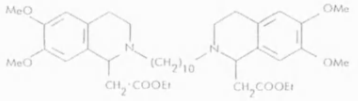

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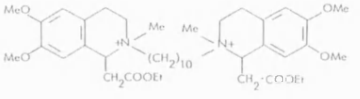

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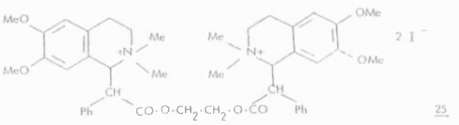
In contrast, but as expected, the rate of elimination of the acid, **28**, was negligible, the UV spectrum being similar to, but quite distinct from that of the ester, **7b**. The acid, **29**, ring-closed, but extremely slowly, the reaction being not quite complete after 10 days at pH 7.4, as judged by the UV absorption spectrum.

As anticipated, the Hofmann elimination reaction at pH 7.4 was highly sensitive to steric factors. Thus the phonylacetate, **13a**, which, like the simpler ester, **7a**, eliminated immediately with a slight excess of dilute alkali, failed to eliminate to any measurable extent in 60 min at pH 7.4. The corresponding cinnamate, **30**, was also stable at pH 7.4. A similar lack of reactivity was exhibited by the methyl-substituted ester, **7c**, which was chosen in order to inhibit recyclisation. Without doubt the presence of the methyl group in the cinnamate, **8c**, would prevent recyclisation, but the slow rate of Hofmann elimination nullified this advantage.



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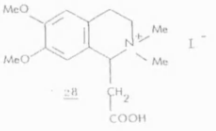

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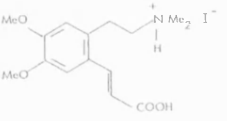

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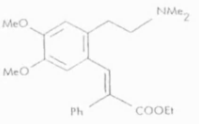

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The bisquaternary derivative, **24**, showed similar Hofmann elimination behaviour to the analogous monoquaternary, **7b**, about 20 % elimination being reached in 18.5 hours. The more complex bisquaternary ester, **25**, like the simple phenylacetate, **13a**, eliminated even more slowly at pH 7.4, elimination being still incomplete after 11 days.

PHARMACOLOGICAL RESULTS AND DISCUSSION

Compounds **7a**, **7b**, **13a**, **13b**, **24** and **25** were tested for neuromuscular blocking activity on the isolated rat diaphragm using (+)-Tubocurarine chloride as standard. Activity in each case was very weak. As expected, the two bisquaternary salts, **24** and **25**, were most active in this test, having activities of 1.7 and 0.7 % of that of (+)-Tubocurarine chloride respectively, and these

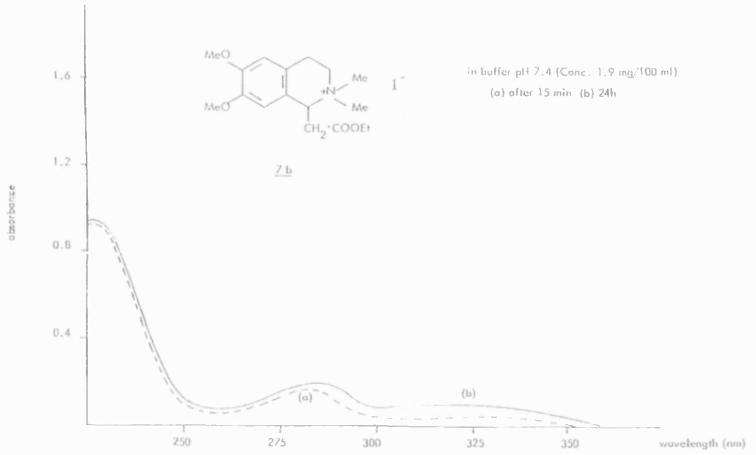

Fig. 1

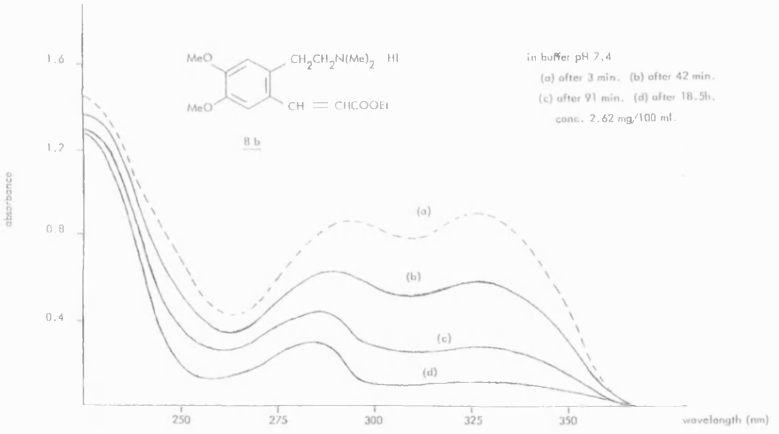

Fig. 2

two compounds were tested *in vivo* on the gastrocnemius muscle - sciatic nerve preparation of anaesthetised cats. Potencies were then 14 and 5.4 % of tubocurarine respectively (Table 1).

The most active compound, **24**, had an action only slightly shorter than that of (+)-Tubocurarine, which may be explained by the finding that the Hofmann elimination reaction is reversible. Ester hydrolysis *in vivo* may contribute to the long action since the resultant acid would not be expected to Hofmann eliminate very quickly, owing to ionisation at physiological pH. The duration of action of the ester-linked bisquaternary, **25**, was also slightly shorter than that of (+)-Tubocurarine, and the block was not antagonised by Edrophonium. In this case presumably ester hydrolysis *in vivo* is slow, since the monoquaternary salts produced by hydrolysis would be expected to be relatively inactive, and Hofmann elimination, although not reversible, is not fast enough to provide a short action. Both compounds, **24** and **25**, caused hypotension and greatly impaired cardiac slowing in response to vagal stimulation.

TABLE 1

Relative neuromuscular blocking potencies (Tubocurarine = 100).

Compound	Rat		Cat
	Potency	Initial potentiation in presence of eserine	
7a	0.5	+++	-
7b	0.4	+++	-
13a	< 0.4	+++	-
13b	0.5	+	-
24	1.7	+	14
25	0.7	+	5.4

EXPERIMENTAL

Melting points were obtained on a Kofler hot-bench and are corrected. Analytical results (C_xH_yN) were within ± 0.3 % of theoretical. IR and proton NMR spectra were obtained for each compound and were in accord with the structures given.

3,4-Dihydroisoquinolinium Salts (**9** and **22**). 3,4-Dihydroisoquinoline and 3,4-dihydro-6,7-dimethoxyisoquinoline were prepared by oxidation of the corresponding tetrahydroisoquinolines with *N*-bromosuccinimide (32) and the resulting bases converted to **9a** and **b** with methyl iodide. 3,4-Dihydro-,6,7-dimethoxy-1-methylisoquinoline was prepared by cyclisation of *N*-acetylhomoveratrylamine using polyphosphoric ester (33) and converted to **9c** with methyl iodide (34). A solution of 1,10-diododecane (40 g, 0.1 mole) and 3,4-dihydro-6,7-dimethoxyisoquinoline (50 g, 0.262 mole) in dry C_6H_6 (100 ml) was heated under reflux for 10 min, cooled and diluted with Et_2O. The precipitate was recrystallised from EtOH - Et_2O to give **22**, as yellow prisms (66 g, 85 %), m.p. 168-170°C. *Anal.* for $C_{32}H_{46}N_2O_4I_2$; C,H,N.

Ethyl 1,2,3,4-Tetrahydro-2-methylisoquinolin-1-yl-acetate (**10a**) *and 1,1'-Bi (1,2,3,4-tetrahydro-2-methylisoquinolinyl)* (**11**). Ethylbromoacetate (3.34 g, 0.02 mole) in dry THF (10 ml) was added over 10 min to a stirred, refluxing mixture of Zn powder (1.31 g, 0.02 mole) and **9a** (5.46 g, 0.02 mole) in THF (20 ml). Refluxing was continued for 30 min, and the mixture cooled, diluted with Et_2O (200 ml) and filtered. The filtrate was washed with H_2O and extracted with 2N HCl. Basification and extraction with Et_2O yielded, after drying and evaporation, an amber oil (2.30 g), containing two substances (TLC) which were separated on a column of silica gel using $CHCl_3$ as solvent. The first to be eluted was **10a** (1.67 g, 36 %), as an amber oil, *Hydrochloride* m.p. 124-125°C from Me_2CO - Et_2O. *Anal.* for $C_{14}H_{20}NO_2Cl$; C,H,N. The second component gave **11** (0.20 g), colourless needles m.p. 129-130°C from H_2O - EtOH. *Anal.* for $C_{20}H_{24}N_2$; C,H,N. The base, **10a**, gave the methiodide, **7a**, as colourless prisms m.p. 112-114°C decomp. from EtOH - Et_2O.

Ethyl 1,2,3,4-Tetrahydro-6,7-dimethoxy-2-methylisoquinolin-1-yl-acetate (**10b**) was prepared as above (19 % yield) from **9b**. The base, **10b**, gave the methiodide, **7b**, as colourless prisms m.p. 172°C from EtOH - Et_2O (lit.[16] m.p. 167-168°C).

1,2,3,4-Tetrahydro-6,7-dimethoxy-2-methylisoquinolin-1-ylacetic acid methiodide (**28**). The ester methiodide, **7b** (20 g) was distilled with 5 % w/v HI (20 ml) over 1.5 h, replacing distillate with H_2O to maintain levels, until 30 ml of distillate was collected. The residue was triturated with AcMe - Et_2O to give a crystalline product which was washed with AcMe - Et_2O, then ether, and dried. Crystallisation from MeOH - Et_2O gave **28** as colourless prisms (1.29 g), m.p. 183-185°C. *Anal.* for $C_{15}H_{22}INO_4$; C,H,N.

Ethyl 1,2,3,4-Tetrahydro-6,7-dimethoxy-1,2-dimethylisoquinolin-1-yl-acetate (**10c**) was prepared as above from **9c** in 15 % yield. The *Methiodide*, 7c, formed colourless prisms, m.p. 158-159°C from EtOH - Et_2O. *Anal.* for $C_{18}H_{28}NO_4I$; C,H,N.

1,10-Decamethylenebis-N,N'-(1-ethoxycarbonylmethyl-1,2,3,4-tetrahydro-6,7-dimethoxyisoquinolinium) dimethiodide (**24**). A mixture of Zn powder (2.60 g, 0.04 mole), ethyl bromoacetate (6.68 g, 0.04 mole) and **22** (3.88 g, 0.005 mole) in dry THF (10 ml) was stirred and heated under reflux for 1 h, cooled, treated with H_2O, and extracted with $CHCl_3$. Evaporation left a pale brown oil which was taken up in Et_2O, filtered, and evaporated to leave the bis tertiary base, **23**, as an oil. The oil was dissolved in CH_3I (10 ml) and refluxed for 30 min. On cooling and dilution with Et_2O to 150 ml a yellow solid separated (1.9 g, 39 %). Pure **24**, m.p. 80-110°C, was obtained by dissolving the solid in hot EtOH, filtering, and adding the solution dropwise to an excess of Et_2O. *Anal.* for $C_{45}H_{66}I_2N_2O_8$; C,H,N.

Ethyl Phenyl (1,2,3,4-tetrahydro-6,7-dimethoxy-2-methylisoquinolin-1-yl)-acetate (**12a**). Ethyl phenylacetate (4.0 g, 0.0245 mole) was added to a solution of Na (0.56 g, 0.0245 mole) in absolute EtOH (15 ml). The solution was refluxed for 10 min, cooled, **9b** (8.12 g, 0.0245 mole) was added, and the mixture heated for an additional 10 min. The EtOH was distilled, H_2O (50 ml) added to the cooled residue, and the product extracted with Et_2O (100 ml). The Et_2O solution was extracted with 2N HCl (2 × 50 ml), the solution basified with 2N NaOH and re-extracted with Et_2O. Drying and evaporation left a gum (4.28 g) which was crystallised (twice) from Et_2O - petrol (b.p. 40-60°C) to give the **12a** (1.64 g, 18 %) as colourless prisms m.p. 107-108°C. *Anal.* for $C_{22}H_{27}NO_4$; C,H,N. The *methiodide*, **13a**, formed pale yellow prisms, m.p. 116-119°C from EtOH - Et_2O. *Anal.* for $C_{23}H_{30}INO_4$; C,H,N.

Ethyl Phenyl (1,2,3,4-tetrahydro-2-methylisoquinolin-1-yl)-acetate (**12b**). The crude product, prepared as above from **9a** (5.46 g, 0.01 mole) and ethyl phenylacetate, was purified by passage through a column of silica gel using $CHCl_3$ as solvent, to give crystalline material (1.95 g) which was recrystallised from petrol (b.p. 40-60°C) to yield **12b** as colourless prisms, m.p. 105-107°C. *Anal.* for $C_{19}H_{23}NO_2$; C,H,N. The *methiodide* **13b** formed colourless prisms, m.p. 144-145°C from EtOH - Et_2O. *Anal.* for $C_{20}H_{26}INO_2$; C,H,N.

Diethyl 1,2,3,4-Tetrahydro-6,7-dimethoxy-2-methylisoquinolin-1-ylmalonate (**14a**). **9b** (6.66 g, 0.02 mole) was added to a solution of diethyl sodio-malonate [prepared from $NaNH_2$ (0.78 g, 0.02 mole) and diethyl malonate (3.20 g, 0.02 mole)] in dry THF (25 ml). After refluxing for 30 min the solution was cooled and diluted with Et_2O to 150 ml, filtered, and evaporated to leave an amber oil. This was redissolved in Et_2O (50 ml), the solution decanted from insoluble material and evaporated to leave **14a** (4.12 g, 56 %).

Attempted Methylation of **14a**.

14a (3.20 g) was dissolved in methyl iodide (15 ml) and allowed to stand for 3 h. Evaporation left a yellow semi-solid residue which was dissolved in hot EtOH. On cooling, the solution slowly deposited **9b** (0.95 g) as yellow prisms, m.p. 199-202°C decomp., undepressed on admixture with authentic material. Dilution of the mother liquor with Et_2O gave a second crystalline fraction (1.26 g) which was recrystallised twice from EtOH - Et_2O to give diethyl 4,5-dimethoxy-2(2-dimethylaminoethyl)-benzylidene malonate methiodide, **18** (0.70 g) as yellow prisms, m.p. 143-146°C. *Anal.* for $C_{21}H_{32}INO_6$; C,H,N.

Dimethyl 1,2,3,4-Tetrahydro-6,7-dimethoxy-2-methylisoquinolin-1-ylmalonate (**14b**) was prepared as for the ethyl ester from dimethyl malonate (2.64 g, 0.02 mole) and **9b** (6.66 g, 0.02 mole), using $NaNH_2$ (0.78 g, 0.02 mole). The crude product was recrystallised from Et_2O - petrol (b.p. 40-60°C) to give **14b** (2.37 g, 35 %) as colourless prisms, m.p. 68-70°C. *Anal.* for $C_{17}H_{23}NO_6$; C,H,N.

Dimethyl 1,2,3,4-Tetrahydro-2-methylisoquinolin-1-ylmalonate (**14c**) was prepared as above from **9a**. The product crystallised from

petrol (b.p. 40-60°C) to give **14c** (80 %) as colourless prisms, m.p. 57-58°C. *Anal.* for $C_{15}H_{19}NO_4$; C,H,N.

1,2-Di-(phenylacetoxy)-ethane (**26**). Phenylacetic acid (136 g) and ethylene glycol (31 g) were heated with C_6H_6 (200 ml) containing H_2SO_4 (0.5 ml) in a Dean-Stark apparatus until no more H_2O was formed. The solution was cooled, shaken with anhydrous Na_2CO_3 (20 g), filtered, and evaporated. The **ester**, **26**, was crystallised from MeOH as colourless prisms (80 g, 54 %), m.p. 45-46°C. *Anal.* for $C_{18}H_{18}O_4$; C,H.

3,6-Dioxa-2,7-dioxo-1,8-diphenyloctamethylene-1,8-bis[1'(6',7'-dimethoxy-1'-2'-3'-4'-tetrahydroisoquinoline)] (**21**) and *3,6-Dioxa-2,7-dioxo-1,8-diphenyloctamethylene-1,8-bis[1'(6',7'-dimethoxy-1',2',3',4'-tetrahydroisoquinolinium)] dimethiodide* (**25**). 1,2-Di-(phenylacetoxy)-ethane (11.92 g, 0.038 mole) in dry THF (40 ml) was stirred and refluxed with $NaNH_2$ (1.56 g, 0.04 mole) for 30 min, cooled, **9b** (13.32 g, 0.04 mole) added, and heated for a further 30 min. After cooling, the mixture was diluted with Et_2O (100 ml) and filtered using more Et_2O (150 ml). The Et_2O solution was washed with H_2O and extracted with 2N HCl (2 × 25 ml), the acid extract rebasified with 2N NaOH, re-extracted with ether, dried and evaporated to leave an oil (6.24 g). This oil was taken up in THF (25 ml), retreated with $NaNH_2$ (0.48 g, 0.0124 mole) and then with **9b** (4.15 g, 0.0124 mole) as above. After extraction as above, the gum (4.5 g) was chromatographed on a column of silica gel with $CHCl_3$ as solvent, giving a gum (3.59 g) which solidified on trituration with Et_2O - petrol (b.p. 40-60°C). Recrystallisation from $CHCl_3$ - petrol (b.p. 80-100°C) gave **27** (0.67 g, 2.5 % overall) as colourless prisms, m.p. 134-135°C (decomp.). *Anal.* for $C_{42}H_{48}N_2O_8$; C_2H_3N. The **dimethiodide**, **25**, formed a pale yellow powder, m.p. 145-147°C, from EtOH - Et_2O. *Anal.* for $C_{44}H_{58}I_2N_2O_8$; C,H,N.

Ethyl 3[2-(2-Dimethylaminoethyl)-4,5-dimethoxyphenyl]-acrylate (**8b**) *hydriodide*. A solution, **7b** (0.5 g, 1.15m mole) in H_2O (20 ml) was basified with 2N NaOH (2 ml). The oil precipitated was extracted with Et_2O, and the Et_2O solution washed with H_2O, dried, filtered, and saturated with dry HI. The dark brown precipitate was recrystallised from EtOH - Et_2O to give **8b hydriodide** (0.31 g, 62 %) as pale yellow plates, m.p. 178-180°C. *Anal.* for $C_{17}H_{26}INO_4$; C,H,N.

3[4,5-Dimethoxy-2-(2-dimethylaminoethyl)-phenyl]-acrylic acid hydriodide (**29**). The methiodide, **7b** (2.0 g, 4.6m mole) was heated with 5N NaOH (5 ml) until homogeneous (15 min). After cooling and dilution with H_2O to 20 ml the solution was acidified with 55 % aqueous HI, giving a brown solid (2.12 g). Recrystallisation from aqueous Me_2CO gave **29** (1.12 g, 60 %) as pale yellow prisms, m.p. 230-231°C (decomp.) lit.[16] 232-234°C.

Ethyl 2-Phenyl-3-[4,5-dimethoxy-2-(2-dimethylaminoethyl)-phenyl]-acrylate (**30**). The methiodide, **13a** (0.79 g) suspended in H_2O (25 ml) was treated with 2N NaOH (5 ml), and the product extracted with ether (50 ml), shaking well for 5 min. The ether layer was washed, dried, evaporated, and the residue crystallised by trituration with petrol (b.p. 40-60°C) to give **30** (0.33 g, 56 %) as colourless prisms, m.p. 80-81°C. The *hydrochloride* gave colourless needles, m.p. 168-170°C, from EtOH - Et_2O. *Anal.* for $C_{23}H_{30}ClNO_4$; C,H,N.

Hofmann elimination studies. UV spectra were recorded on an automatic recording spectrophotometer (Unicam SP 800), using the « fast scan » mode, commencing at the long wavelength end of the spectrum. Solutions were prepared by adding M/15 phosphate buffer, pH 7.4, to the compound (0.5 — 1.0 mg, accurately weighed), and adjusting to 25.00 ml with buffer. Bisquaternary salts were dissolved in EtOH (1 ml) before adjusting to volume with buffer solution, since in buffer alone the rate of dissolution was very slow. The effect of the EtOH on pH was negligible.

Neuromuscular blocking potency.

(a) *Rat phrenic-nerve diaphragm*. Duplicate preparations from male Wistar rats (130-150 g) were set up as described by Bülbring (35) in parallel in Krebs-Henseleit (36) solution (100 ml), bubbled with oxygen containing 5 % CO_2 and maintained at 35°C. The phrenic nerve was threaded through a thin rubber diaphragm into a small glass tube filled with solution and containing one electrode, the other electrode being in contact with the bath fluid. Maximal twitches were produced by stimulation at supramaximal voltage, using square wave pulse of 0.2 m sec duration for the nerve and 2 m sec for the muscle, normally at a frequency of 1 shock per 5 sec. Contractions of the diaphragms were recorded by isotonic levers on a smoked drum.

(b) *Cat gastrocnemius muscle - sciatic nerve*. Cats of either sex, weighing between 2.8 and 4.2 kg were used. Anaesthesia was induced was induced with 4-8 % halothane and maintained with chloralose (60-80 mg/kg i.v.) after cannulation of a jugular vein. The right sciatic nerve was exposed between the thigh muscles, crushed centrally and placed on shielded bi-polar platinum electrodes. The hind limb was fixed securely to a rigid frame by a steel drill through the tibia or femur at the knee and a clamp at the ankle. The limb was maintained at 37°C by radiant heat. The tendon of the right gastrocnemius muscle was cut and attached to a Grass FT 10 force displacement transducer. The sciatic nerve was stimulated at a frequency of 0.1 Hz with rectangular pulses of supramaximal voltage and 0.05-0.1 m sec duration. Recordings were made on a Beckman type R Dynograph. The oesophagal temperature was monitored with a termistor probe (Yellow Spring Instruments) and body temperature was maintained at 36-37°C with a temperature controller and rectal probe (Southern Scientific). Drugs were given i.v. and, during neuromuscular paralyses, the lungs were ventilated with air using a Starling Ideal pump (rate 20/min, tidal volume 40-50 ml) ; arterial blood-gas tensions were maintained within normal limits. Test drugs were compared (+)-tubocurarine chloride.

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RÉSUMÉ

Une nouvelle approche pour la préparation d'agents bloquants neuromusculaires à action courte est examinée. Elle est modelée sur la dégradation simple selon Hofmann de l'alcaloïde quaternaire pétaline, 5. Une série de composés de l'éthoxycarbonylmethyl-1-tétrahydroisoquinolinium et polyméthylène *bis*-éthoxycarbonylméthyl-1-tétrahydroisoquinolinium a été préparée. Ces composés ont des effets bloquants neuromusculaires faibles, et subissent seulement une élimination partielle suivant Hofmann *in vitro* à pH physiologique. Les produits d'élimination subissent une rétro-réaction d'Hofmann, et un équilibre avec le sel quaternaire s'établit lentement.

ZUSAMMENFASSUNG

Ein neuer Weg zur Herstellung von im Stoffwechsel abbaubaren, kurz wirkenden neuromuskulär-blockierenden Verbindungen wurde untersucht, der auf dem leichten Hofmann'schen Abbau des quaternären Alkaloids Petalin 5 basiert. Eine Reihe von 1-Ethoxycarbonylmethyltetrahydroisochinolinium- und Polymethylen-bis-1-ethoxycarbonylmethyltetrahydroisochinolinium- Verbindungen wurde hergestellt. Die Verbindungen hatten nur geringe neuromuskulär-blockierende Aktivität und unterlagen *in vitro* bei physiologischem pH nur einer unvollständigen Hoffmann-Eliminierung. Die Eliminierungsprodukte gehen eine retro-Hoffmann-Reaktion ein und das Gleichgewicht mit dem quaternären Salz wird nur langsam erreicht.

REFERENCES

(1) V.P. Witticker and S. Wijesundera, *Biochem. J.*, 1952, **52**, 475.
(2) F.I. Tsuji and F.F. Foldes, *Fed. Proc.*, 1953, **12**, 374.
(3) H. Lehman and E. Silk, *Brit. Med. J.*, 1953, **1**, 763.
(4) F.F. Foldes and F.I. Tsuji, *Fed. Proc.*, 1953, **12**, 321.
(5) F.F. Foldes, P.G. McNall and J.H. Birch, *Brit. Med. J.*, 1954, **1**, 967.
(6) F. Roth and H. Wüthrick, *Brit. J. Anaesth.*, 1969, **41**, 311.
(7) H.C. Churchill-Davidson, *Brit. Med. J.*, 1954, **1**, 746.
(8) J.A. Mathias and C.D.G. Evans Prosser, *Prog. Anaesth.*, 1970, 1153.

(9) F.T. Evans, P.W.S. Gray, H. Lehman and E. Silk, *Lancet*, 1953, **1**, 1229.
(10) F.T. Evans, P.W.S. Gray, H. Lehman and E. Silk, *Brit. med. J.*, 1953, **1**, 136.
(11) F.F. Foldes, *Muscle Relaxants in Anaesthesiology*, Thomas, Springfield, Illinois, 1957, **52**.
(12) R. Fusco, G. Palazzo, S. Chiavarelli and D. Bovet, *Gazz. Chim. Ital.*, 1949, **79**, 836.
(13) F.M. Carey, J.J. Lewis, J.B. Stenlake and W.D. Williams, *J. Pharm. Pharmacol.*, 1961, **13**, 103T.
(14) M.T. de Cournout, B. Dupuy and J. Jacquenoud, *Agressologie* 1961, **3**, 627.
(15) P.G. Waser and P. Harbeck, *Anaesthetist*, 1962, **11**, 33.
(16) W.R. Buckett and I.L. Bonta, *Fed. Proc.*, 1966, **25**, 718.
(17) W.R. Buckett, C.L. Hewett and D.S. Savage, *Chim. Ther.*, 1967, **2**, 186.
(18) W.R. Buckett, C.E.B. Marjoribanks, F.A. Marwick and M.B. Morton, *Brit. J. Pharmacol.*, 1968, **32**, 671.
(19) B.R. Simpson, T.M. Savage, E.I. Foley, L.A. Ross, L. Strunin, B. Walton, M.P. Maxwell and D.M. Harris, *Lancet*, 1972, *i*, 516.
(20) R. Hughes and D.J. Chapple, *Brit. J. Anaesth.*, 1976, **48**, 59.
(21) N.J. McCorkindale, D.S. Magrill, M. Martin-Smith, S.J. Smith and J.B. Stenlake, *Tetrahedron Lett.*, 1964, **51**, 3841.
(22) A. Brossi, L.H. Chopard-dit-Jean, J. Würsch and O. Schneider, *Helv. Chim. Acta*, 1960, **43**, 583.
(23) K. Bernauer, *Helv. chim. Acta*, 1968, **51**, 1120.
(24) W.M. Whaley and T.R. Govindachari, *Org. Reactions*, 1951, **6**, 74.
(25) W.M. Whaley and T.R. Govindachari, *Org. Reactions*, 1951, 6, 151.
(26) M. Freund and G. Bode, *Ber.*, 1909, **42**, 1746.
(27) H. Schmidt and P. Karrer, *Helv. chim. Acta*, 1949, **32**, 960.
(28) R.L. Shriner, *Org. Reactions*, 1942, **1**, 1.
(29) B.B. Dey and T.R. Govindachari, *Proc. Natl. Inst. Sci., Indio*, 1940, **6**, 219 (*Chem. Abstr.*, 1942, **36**, 5178).
(30) P. Cerutti and H. Schmidt, *Helv. chim. Acta*, 1964, **47**, 203.
(31) C. Liebermann and A. Glawe, *Ber.*, 1904, **37**, 2738.
(32) E. Eckhart, *Magy. Kem. Folyoirat*, 1964, **70** (7), 296-9 (*Chem. Abstr.*, 1964, **61**, 13335).
(33) Y. Kanoaka, E. Sato, O. Yanemitsu and Y. Ban, *Tetrahedron Lett.*, 1964, **35**, 2419.
(34) Y. Ban, O. Yanemitsu and M. Terashima, *Chem. Pharm. Bull.* (Tokyo), 1960, **8**, 194 (*Chem. Abstr.*, 1961, **55**, 7455).
(35) E. Bülbring, *Brit. J. Pharmacol. Chemother.*, 1946, **1**, 38.
(36) H.A. Krebs and K. Henseleit, *Hoppe-Seyl. Z.*, 1932, **210**, 33.

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Biodegradable neuromuscular blocking agents II. Quaternary ketones

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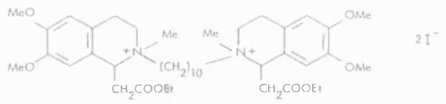
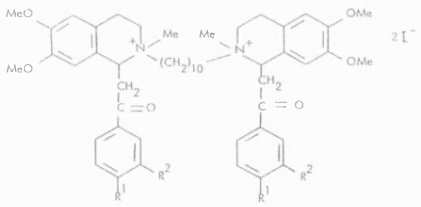
Quaternary 1-phenacyl-1,2,3,4-tetrahydroisoquinolinium salts underwent reversible Hofmann elimination at pH 7.4. The extent of elimination (up to 90 %) was dependent on the nitrogen substituents (Me, Et or decamethylene), and the rate of elimination was largely governed by the phenacyl ring substituents (H, OMe or Cl). Although Hofmann elimination in some cases proceeded very rapidly in buffer at pH 7.4, the compounds were long-acting neuromuscular blocking agents and of low potency.

We have outlined the rationale behind a new approach to the preparation of short-acting neuromuscular blocking agents, based on Hofmann elimination at physiological pH (1). In that paper we described the preparation and properties of esters such as **1**, which show moderate neuromuscular blocking activity, with a slow but reversible Hofmann elimination at pH 7.4. With the objective of improving the rate and extent of Hofmann elimination, and hence shortening the block, we have now extended the study to the phenyl ketones, **2**, which show a resemblance to laudexium, **3**, a long-acting but otherwise effective neuromuscular blocking agent (2).

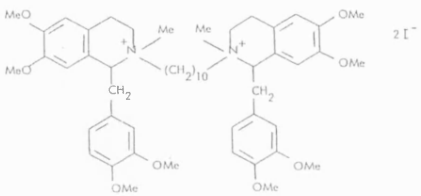


1



2

	R^1	R^2
a	H	H
b	OMe	H
c	OMe	OMe
d	Cl	H



3

The methylene protons adjacent to the carbonyl group are more acidic in the ketones than in the esters tested previously. Hence, more rapid Hofmann elimination was anticipated. The acidity of these protons can also be influenced by alteration of the substituents on the phenyl ring, so that, additionally, the rate of Hofmann elimination can be manipulated in this way.

CHEMISTRY

A series of monoquaternary salts, **7**, in which the ring and nitrogen substituents were varied, were first prepared as model compounds. The tertiary bases, **6**, were synthesised by base catalysed addition of substituted acetophenones, **4**, to 3,4-dihydroisoquinolinium methiodides, **5** (3, Scheme 1) and the method was later applied to decamethylene *bis*-isoquinolines without difficulty. Quaternisation of the bases with methyl iodide proceeded normally, except in the case of the simplest derivative, **7a**, where the crude salt appeared to have the correct structure, but on

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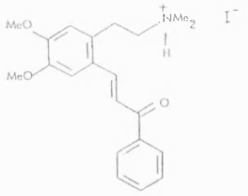
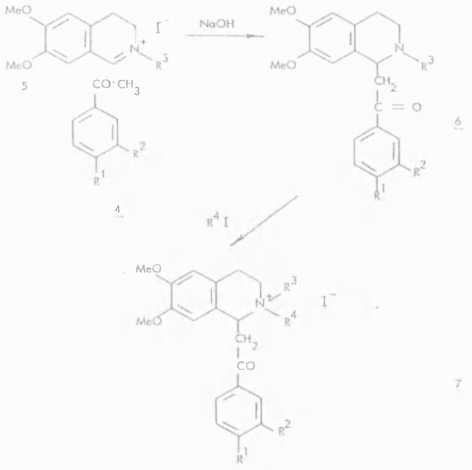


8

crystallisation from n-propanol underwent spontaneous Hofmann elimination to give the methine base hydriodide, **8**. Attempted quaternisation of the *bis*-isoquinolines with ethyl iodide was unsuccessful, giving intractable, oily products.

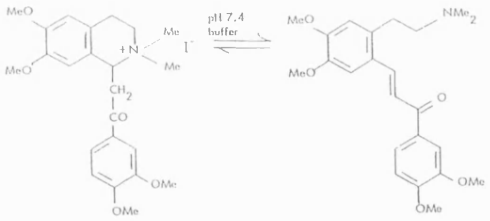


	R^1	R^2	R^3	R^4
a	H	H	Me	Me
b	OMe	OMe	Me	Me
c	OMe	H	Me	Me
d	Cl	H	Me	Me
e	OMe	OMe	Et	Me
f	OMe	OMe	Et	Et

Scheme I

HOFMANN ELIMINATION STUDIES AND PHARMACOLOGICAL RESULTS

Uv spectra were used to follow the Hofmann elimination (Scheme 2) in buffer at pH 7.4, as described previously (1). The half-time to reach equilibrium and the percentage elimination (Table I) were based on visual estimates from the uv spectra. The extent of elimination was judged after equilibrium was reached for each compound by comparison with a spectrum of the methine base obtained by treating an aqueous solution of the quaternary salt with a slight excess of sodium hydroxide. Under these conditions of high pH, Hofmann elimination was immediate, as with the esters investigated previously (1) and was assumed to be complete, since in cases where the methine base was isolated and characterised, the uv spectrum was identical with that obtained as described.



Scheme II

It may be seen from Table I that Hofmann elimination was much more rapid with the ketones than with the esters testeds previously (data for the estor, **1**, are given for comparison). Compounds **2a-d** were all of low potency when tested on the rat phrenic nerve-diaphragm preparation, and, despite rapid and almost complete (90 %) Hofmann elimination showed no evidence of being short-acting. Their action, however, was not antagonised by physostigmine.

TABLE I

COMPOUND	HOFMANN ELIMINATION at pH 7.4		NEUROMUSCULAR BLOCKING POTENCY
	Half-time to reach equm. (min)	Extent of Elimination (%)	d-Tubocurarine = 100
1	30	20	1.7
7a*	4	10	-
b	4	10	-
c	3.5	10	-
d	1.5	15	-
e	7.5	55	-
f	1.5	25	-
2a	5	90	1.3
b	7	90	0.7
c	11	90	0.7
d	2.5	90	0.5

(*) Rat phrenic nerve - diaphragm preparation.

(*) Crude material. Attempts to recrystallise this material resulted in its conversion to the isomer **6**.

Clearly, the effect of electron-donating (OMe) groups has a retarding effect on the rate of Hofmann elimination, whereas an electron-withdrawing (Cl) substituent increases the rate of elimination to a point where, if elimination were complete and the other requirements for potency were satisfied (1) neuromuscular block would be very short. It has thus been shown that Hofmann elimination is in theory a viable basis for shortening drug action, and that compounds which undergo rapid Hofmann elimination in buffer at pH 7.4 are not necessarily too unstable to be obtained in a pure state.

The effect of increased bulk at the quaternary centre was mainly to increase the equilibrium concentration of methine base, an effect which is understandable since steric strain will be relieved in the open-chain form. It seems probable that the quaternisation of the decamethylene bis-isoquinolines with ethyl iodide, which failed, would have given compounds exhibiting virtually complete elimination at pH 7.4. Many attempts at quaternisation of similar bases with ethyl iodide have also failed (4), apparently because the relatively slow rate of quaternisation allows the base present in solution to catalyse the Hofmann elimination of any quaternary salt formed, giving a mixture of products.

In order to be certain that these ketones, like the esters (1), undergo a reversible Hofmann reaction, we studied the behaviour at pH 7.4 of the methine base obtained by treatment of **7b** with excess NaOH. As expected, the spectum of the methine base changed on standing in buffer pH 7.4 and after equilibration was identical with that obtained directly from the quaternary salt in the region above 250 nm unaffected by the anion, the equilibrium position corresponding to about 10 % elimination.

EXPERIMENTAL

Melting points were obtained on a Kofler hot-bench, and are corrected. Analytical results (C,H,N) were within ± 0.3 % of theoretical. Ir and proton NMR spectra were obtained for each compound and were in accord with the structures given.

3,4-Dihydroisoquinolinium Salts **5**. 3,4-Dihydro-6,7-dimethoxy-2-isoquinolinium iodide, **5** (R^3 = Me) and decamethylenebis (3,4-dihydro-6,7-dimethoxyisoquinolinium iodide) were prepared as described previously (1). A solution of 3,4-dihydro-6,7-dimethoxyisoquinoline (32 g) in EtOH (50 ml) and ethyl iodide (50 ml) was kept at room temperature overnight, then diluted with Et_2O to 500 ml. Recrystallisation of the precipitate, from EtOH-Et_2O gave **5** (R-Et) (51 g, 87 %) as yellow prisms, m.p. 188-189°C. *Anal.* for $C_{13}H_{18}NO_2I$; C,H,N.

2-Alkyl-1,2,3,4-tetrahydro-6,7-dimethoxy-1-phenacylisoquinolines **6**. These compounds were prepared by condensing the appropriate acetophenone **4** and 3,4-dihydroisoquinolinium salt **5** in the presence of alkali, by the general method **3** (Table II). *Anal.* for $C_{13}H_{18}NO_2Cl_2$, 0.5 H_2O ; C,H,N.

2,2-Dialkyl-1,2,3,4-tetrahydro-6,7-dimethoxy-1-phenacylisoquinolinium iodides **7**. A solution of the tertiary base, **6** (1.0 g) in the alkyl halide (5 ml) was allowed to stand at room temperature. After 15 min (24 h when using ethyl iodide), ether (75 ml) was added to precipitate the crude product. Recrystallisation gave the pure isoquinolinium salts (Table III), except in the case of **7a**. In this case, the crude product appeared to have the required cyclic structure. However, recrystallisation from n-PrOH gave the isomer *2-(2-dimethylaminoethyl)-4,5-dimethoxybenzylidene-acetophenone hydriodide*, **6**, as a bright yellow powder, m.p. 250-252°C. *Anal.* for $C_{21}H_{28}NO_3I$; C,H,N.

TABLE II

6			m.p.		m.p. of HCl salt	
R^1	R^2	R^3	Found	lit.[3]	Found	lit.[3]
H	H	Me	80°	80 - 81°	150°	-
OMe	OMe	Me	oil	-	120 - 122°	120°
OMe	H	Me	84°	91 - 92°	-	120°
Cl	H	Me	104 - 105°	106 - 107°	-	151 - 152°
OMe	OMe	Et	oil	-	124 - 126° *	-

(*) *Anal.* for $C_{23}H_{30}NO_5Cl$, 0.5 H_2O ; C,H,N.

TABLE III

7	m.p.	Recrystallation Solvent	Formula (Anal.: C,H,N)
a	-	- *	$C_{21}H_{28}NO_3I$
b	146 - 150°	MeOH — Et_2O	$C_{23}H_{30}NO_5I \cdot \frac{1}{2}H_2O$
c	139 - 145°	n-PrOH	$C_{22}H_{28}NO_4I$
d	140 - 148°	EtOH — Et_2O	$C_{21}H_{25}NO_3ClI$
e	180 - 182°	$CHCl_3$ — Et_2O	$C_{24}H_{32}NO_5I$
f	123 - 125°	n-PrOH	$C_{25}H_{34}NO_5I \cdot H_2O$

(*) See text.

Hofmann reaction of 1,2,3,4-tetrahydro-6,7-dimethoxy-2,2-dimethyl-1-(3,4-dimethoxyphenacyl) isoquinolinium iodide **5b**. A suspension of the quaternary salt (3.00 g) in water (25 ml) was basified with 2N sodium hydroxide (10 ml). After shaking thoroughly, the mixture was extracted with ether (150 ml total) which was washed with water, dried (Na_2SO_4), and evaporated to leave an amber oil (2.09 g). With ethereal hydrogen chloride this furnished *2-(2-dimethylaminoethyl)-4,5-dimethoxybenzylidene-3,4-dimethoxyacetophenone hydrochloride* which formed pale yellow prisms (1.23 g, 50 %), m.p. 178-181°C, from ethanol/ether. *Anal.* for $C_{23}H_{29}NO_5Cl$, 0.5 H_2O ; C,H,N.

Decamethylenebis(1-phenacylisoquinolinium iodides) **2**.

Example : *decamethylenebis(1,2,3,4-tetrahydro-6,7-dimethoxy-2-methyl-1-(3,4-dimethoxyphenacyl) isoquinolinium iodide)* **2c**. 3N NaOH (3.85 ml) was added to a solution of decamethylenebis(3,4-dihydro-6,7-dimethoxyisoquinolinium iodide) (5.00 g) and acetoveratrone (2.90 g) in methanol (50 ml). The solution was kept at room temperature overnight, then evaporated. The residue was digested with ethyl acetate (100 ml), which was washed with brine, dried (Na_2SO_4), and evaporated to leave an amber gum

(6.22 g). This material was purified, using a column of neutral alumina and ethyl acetate as eluting solvent, to give the bis(tertiary base) (4.00 g, 71 %) as an amber oil. A solution of this material (1.00 g) in chloroform (3 ml) and methyl iodide (3 ml) was allowed to stand at room temperature overnight, then diluted with ether (150 ml). The precipitated solid was dissolved in hot ethanol (15 ml) and this solution was filtered and added dropwise to ether (150 ml). The precipitated solid was collected, washed with ether, and dried to give decamethylenebis(1,2,3,4-tetrahydro-6,7-dimethoxy-2-methyl-1-(3,4-dimethoxyphenacyl) isoquinolinium iodide), **2c** (see Table IV) as a pale yellow powder. The other compounds in the series were prepared and purified similarly.

TABLE IV

2	m.p.	Formula (Anal. C,H,N)	Yield (%)
a	148 - 150°	$C_{50}H_{66}N_2O_6I_2\ 2H_2O$	51
b	154 - 158°	$C_{52}H_{70}N_2O_8I_2\ H_2O$	42
c	140 - 142°	$C_{54}H_{74}N_2O_{10}I_2\ H_2O$	45
d	139 - 142°	$C_{50}H_{64}N_2O_6Cl_2I_2\ H_2O$	43

Hofmann elimination studies were carried out as previously described (1).

Neuromuscular blocking potency was measured on the rat phrenic nerve-diaphragm preparation as previously described (1).

ACKNOWLEDGEMENTS. — We thank the U.K. Medical Research Council for financial assistance.

RÉSUMÉ

Les sels quaternaires de phénacyl-1-tétrahydro-1,2,3,4-isoquinolinium subissent une élimination d'Hofmann réversible à pH 7,4. Le degré d'élimination (jusqu'à 90 %) dépend des substituants de l'azote (Me, Et ou décaméthylène), et la vitesse d'élimination dépend largement des substituants du cycle phénacyle (H, OMe ou Cl). Bien que l'élimination d'Hofmann se fasse, dans certains cas, très rapidement dans un tampon à pH 7,4, les composés sont des agents bloquants musculaires à action longue et puissance faible.

ZUSAMMENFASSUNG

Quaternäre 1-Phenacyl-1,2,2,4-tetrahydroisochinolinium-Salze unterliegen bei pH 7,4 einer reversiblen Hofmann-Eliminierung. Das Ausmaß der Eliminierung (bis zu 90 %) hängt von den Substituenten am Stickstoff (Me, Et oder decamethylen). Die Geschwindigkeit der Eliminierung wird weitgehend beeinfluß durch die Substituenten am Phenacylring (H, OMe oder Cl). Obwohl die Hofmann-Eliminierung in einigen Fällen im Puffer bei pH 7,4 sehr schnell erfolgte, waren die Verbindungen von langdauernder neuromuskulär blockierender Wirkung und geringer Aktivität.

REFERENCES

(1) J.B. STENLAKE, John URWIN and R.D. WAIGH (preceding paper). *Eur. J. Med. Chem.*, 1979, **14**, 77.
(2) W.C. BOWMAN, *Progress in Medicinal Chemistry*, 1962, **2**, 122.
(3) Belg. Pat. 619,513 (*Chem. Abst.*, 1963, **59**, 11453d).
(4) Unpublished data from these laboratories.

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Biodegradable neuromuscular blocking agents.

Part 3. Bis-quaternary esters

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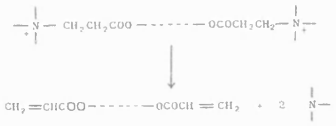
Two series of bis-quaternary ammonium salts bearing ester carbonyl moieties adjacent to the β-carbon of the ammonium group and designed to undergo Hofmann elimination *in vivo* have been synthesised and tested as potential neuromuscular blocking agents in cats. Variations in duration of action, potency and vagal block are reported and related to structure.

In previous papers (1, 2), we have outlined our ideas for producing biodegradable neuromuscular blocking agents by utilising the rapid Hofmann elimination of certain quaternary ammonium salts to bring about their destruction *in vivo* without the intervention of enzymes. Our earlier efforts were unsuccessful (1, 2) because after Hofmann elimination, both moieties remained attached to the same residue, and an unpredicted retro-reaction occurred to re-form the quaternary salt, producing an equilibrium mixture with a long duration of action.

Two ways of overcoming the problem, while retaining the same theoretical basis of Hofmann elimination in bis-quaternary ammonium salts, are represented generally for the esters in Schemes 1 and 2. In Scheme 1, we utilised two tetrahydroisoquinoline units linked through the 1-position, in the hope of obtaining potent non-depolarising agents by analogy with some *NN*-linked compounds (3). Unfortunately, the bis-quaternary salts **1** that we obtained were not short-acting, perhaps because of ester hydrolysis, which slows Hofmann elimination and leaves the quaternary centres intact. The products also showed vagal blockade. We, therefore, concentrated our efforts on a thorough investigation of salts having the basic structure of Scheme 2. This offers the advantage that ester hydrolysis also destroys the bis-quaternary structure to give two mono-quaternary structures which would be expected to have negligible paralysing activity, as in Suxamethonium.

Scheme 1



Scheme 2

CHEMISTRY

The salts, **1a-1d** and **7a-7f**, were prepared by the routes shown in Schemes 3 and 4 respectively. These salts are quaternary derivatives of Michael addition products between bis-amines, **2**, and methyl acrylate (Scheme 3), or certain mono-amines **4** and ethylene glycol diacrylate, **3** (Scheme 4).

The necessary 1-substituted 1,2,3,4-tetrahydro-6,7-dimethoxyisoquinolines **2** were obtained by Bischler Napieralski (4, 5) cyclisation of appropriate amides **12** to 3,4-dihydroisoquinolines **13**, followed by sodium borohydride reduction. Tetrahydropapaverine, **4** (R = 3,4-dimethoxybenzyl) was prepared by a modification (6) of the method described by TAYLOR (7) in which papaverine is reduced by zinc and hydrochloric acid. 1,2,3,4-Tetrahydro-6,7-dimethoxyisoquinoline **4** (R = H) was prepared by a Pictet Spengler (8, 9) synthesis using homoveratrylamine hydrochloride and formaldehyde. In some initial

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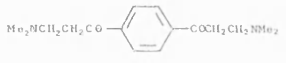

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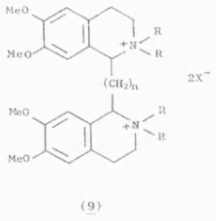

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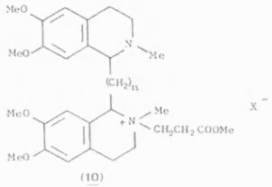
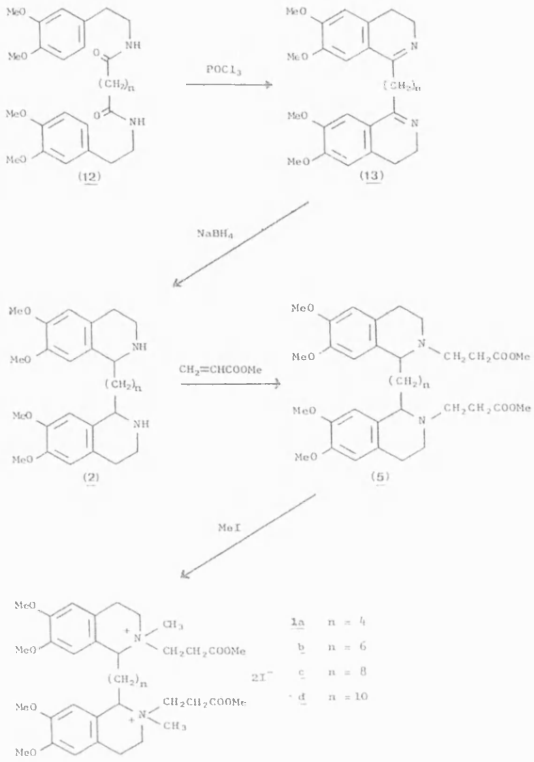


(10)

$R_3\overset{+}{M}eNCH_2CH_2COOCH_2CH_2OCOCH_2CH_2\overset{+}{N}MeR_3 \cdot 2I^-$

(11a, R = Me)
(11b, R = Et)

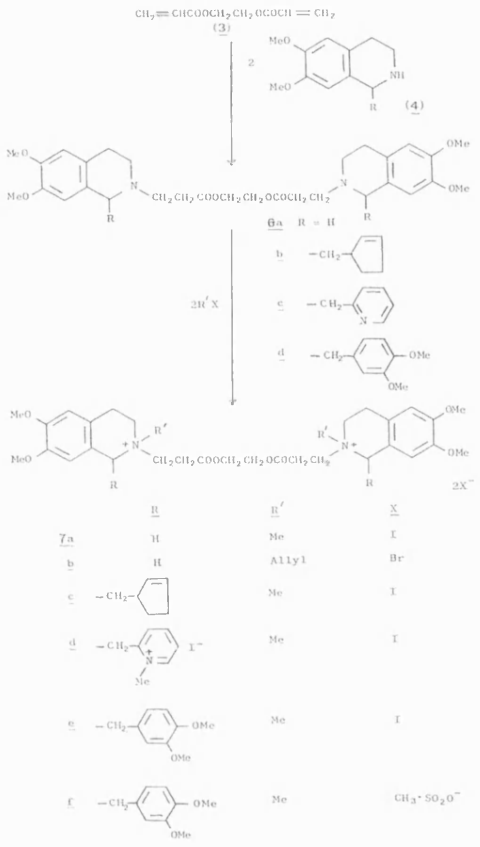


Scheme 3

experiments, an excess of formaldehyde was employed in this preparation and, under these conditions, basification of the mixture at the end of the reaction gave only bis-(1,2,3,4-tetrahydro-6,7-dimethoxyisoquinolin-2-yl)methane. This *gem*-diamine has been previously reported (10) from an alternative synthesis. Its formation was avoided in the present work by maintaining acidic conditions (11) throughout the preparation.

Michael addition of all secondary amines to appropriate acrylate esters proceeded smoothly to yield the corresponding tertiary amines, **5** and **6**. The Michael bases, **5** and **6**, were quaternised by methyl iodide to the corresponding salts, and the bis-amine (**6a**, Scheme 4) was also successfully quaternised by allyl bromide to give compound (**7b**, Scheme 4). Successful quaternisation of Mannich and Michael amines (where a carbonyl function is positioned β to the amino group) by methyl iodide is well documented (1, 2, 12-14). Less reactive alkyl halides than methyl iodide and more reactive, but bulkier, halides, such as benzyl bromide (15), may quaternise such bases with accompanying *in situ* Hofmann elimination, quaternised molecules in the system being Hofmann-eliminated by the basic, unquaternised amino functions still present.

The factors affecting the incidence of such *in situ* elimination seem to be the steric requirement of the base being quaternised, the steric requirement (16) and reactivity of the alkylating agent, and the acidity of the β-protons in the resulting quaternary compound. The importance of steric features in the alkylating agent is illustrated from our attemps to quaternise Michael amines with benzyl bromide. These have repeatedly shown *in situ* Hofmann elimination whereas allyl bromide, with relatively unhindered amines at least, has shown no such tendency, despite the fact that the former is reported to be three times more powerful as an alkylating agent than the latter in other systems (15). Where the β-proton in the resultant quaternary is particularly acidic, *in situ* elimination even accompanies quaternisation using methyl iodide and other powerful methylating agents such as dimethyl sulphate and methyl methanesulphonate. Thus, the bis-amino diketone, **8**, gave tetramethylammonium iodide and trimethylammonium iodide as the only nitrogenous products on treatment with methyl iodide (17). Similar results were obtained with methyl methanesulphonate. We have also found that, to a limited extent, elimination can accompany the quaternisation of sterically-hindered 1-benzyltetrahydroisoquinoline bis-amines, such as **6*d***, with methyl iodide and



Scheme 4

methyl methanesulphonate, and precautions have to be taken to minimise this tendency by using a large excess of methylating agent.

It is clear that where *in situ* elimination accompanies quaternisation of bis-amine Michael addition products, the resultant mixture is complex. Complete Hofmann elimination of the pure bis-quaternary salts, **7**, however, will give exactly two mole equivalent of tertiary amine and one mole equivalent of acrylate. Such decomposition has been applied in the characterisation of **7a** using thin-layer and gas-liquid chromatography.

PHARMACOLOGICAL RESULTS AND DISCUSSION

Neuromuscular blocking potency and the extent of vagal block were measured in anaesthetised cats. The results are given in Tables I and II. Potencies of the αω-bis-isoquinolin-1-ylalkanes (**1**, Table I) were consistently of

TABLE II

Mean neuromuscular blocking potencies and vagolytic effects of 1,10-bis-amino-4,7-dioxa-3,8-dioxodecane quaternary salts, **7** *and* **11**, *in cats*

Compound No.	Relative molar neuromuscular blocking potency[1]	PD50[3]	VD50[4]	VD50/PD50
7a	6(2)[2]	2.2	2.2	1.0
b	0[5](2)	-	0.5	-
c	12(2)	1.4	1.9	1.4
d	15(2)	1.4	1.0	0.7
e	58(2)	0.36	2.6	7.2
11a	420[6](2)	-	-	-
b	17(2)	0.46	2.4	5.2

[1] Tubocurarine = 100.
[2] No. of animals.
[3] Dose in mg/kg iv producing 50 % neuromuscular blockade.
[4] Dose in mg/kg iv producing 50 % vagal blockade.
[5] No block recorded at doses up to 2 mg/kg.
[6] Relative to Suxamethonium Chloride ; depolarising block as contracture on isolated chick biventer-cervicis preparation.

TABLE I

Mean neuromuscular blocking potencies and vagolytic effects of αω-bis[1,2,3,4-tetrahydro-6,7-dimethoxy-2-(2-methoxycarbonylethyl)isoquinolin-1-yl]alkane dimethiodides **1** *in cats*

Compound No.	n	Relative molar neuromuscular blocking potency[1]	PD50[3]	VD50[4]	VD50/PD50
1a	4	0[5](2)[2]	-	4.6	-
b	6	20(3)	0.86	4.4[6]	5.1
c	8	20(2)	0.84	1.6	1.9
d	10	22(2)	0.77	1.6	2.1

[1] Tubocurarine = 100.
[2] No. of animals.
[3] Dose in mg/kg iv producing 50 % neuromuscular blockade.
[4] Dose in mg/kg iv producing 50 % vagal blockade.
[5] No block recorded at doses up to 4 mg/kg.
[6] Calculated by extrapolation.

a low level. Compound **1a** was inactive in cats as a neuromuscular blocking agent, while compounds **1b-1d** all had similar potencies about 20 % of that of Tubocurarine. On the other hand, vagal blockade was greater in the longer chain compounds, **1c** and **1d**, than in **1b**.

In view of the substantial anticholinesterase activity (18) of the related compound **9**, the pharmacological observations in the present series may be the result of some complex pharmacodynamics. Also, *in vivo* Hofmann elimination could occur stepwise. The resulting mono-quaternary compound, **10**, like Tubocurarine, would then possess substantial neuromuscular blocking activity. This factor could, therefore, contribute to the disappointingly long action of compound **1b** (recovery from onset of full block *ca* 70 min). Such a possibility is quite clearly much less likely in compounds which fragment according to Scheme 2, and consequently we have directed the bulk of our efforts in that direction.

Several compounds, which formally accord with the bis-quaternary structure in Scheme 2 have already been described (14, 19, 20). The reversed succinylcholine ester, **11a**, has been reported to be more potent than Suxamethonium (19). This has been confirmed in the present work, and the compound shown to produce a depolarising block with contracture in the chick biventer cervicis preparation (Table II). Substitution of two methyls on each nitrogen by ethyl produced a non-depolarising agent, **11b**, as expected (21, 22), but potency was reduced (19). There was also some vagal block, one of the major disadvantages associated with most competitive neuromuscular blocking agents (23).

Introduction of a 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline nucleus in the bis-quaternary compound, **7a**, following the work of TAYLOR (3), resulted in a duration of action (recovery from onset of full block *ca* 14-20 min) shorter than that of **1b** and some reduction in vagal blockade. Potency was also reduced. Attempts to shorten duration of action in the *N*-allyl compound, **7b**, as in Alcuronium (24), resulted in complete loss of activity, and an increase in vagal block. We, therefore, turned our attention to tetrahydroisoquinolines with a bulky 1-substituent (Table II), which in earlier work had reduced side-effects while maintaining potency (25). Of the three substituents used, the 3,4-dimethoxybenzyl group, as in **7c**, gave by far the best results with respect to potency and a wide separation between neuromuscular and vagolytic effects ; full recovery from onset of 100 % paralysis occurred in 20 min. Detailed accounts of the activity of this and closely-related analogues and homologues are presented in Part 4 of this series.

EXPERIMENTAL

Unless stated otherwise, melting points were recorded on a Kofler Heizbank 184321 melting point apparatus. Infrared spectra were obtained on a Perkin-Elmer 157 instrument using either liquid films or in KCl discs (for solids). Proton magnetic resonance spectra were recorded on a Perkin-Elmer R12 instrument operating at 60 MHz. TMS was used as internal standard. IR and proton NMR spectra were in accord with the structures given and are available from the authors (RDW and GHD) on request. Microanalytical results (CHN, except where otherwise stated) were within ± 0,4 % of theoretical.

N-[2-(3,4-Dimethoxyphenyl)ethyl]cyclopent-2-enylacetamide

Homoveratrylamine (19 g ; 0.105 mole) and cyclopent-2-enylacetic acid (12.6 g ; 0.1 mole) were heated together in an open vessel at 190-200°C for 2 h. The dark oil remaining on cooling was dissolved in ethyl acetate, and the solution was then boiled with charcoal and filtered. To the filtrate, warmed to reflux temperature, was added sufficient light petroleum (b.p. 60-80°C) to deposit an almost black, viscous oil. The liquid was decanted from the oil and refrigerated for 18 h. The *amide* (11.3 g ; 39 %) crystallised as long, fine needles from light petroleum (b.p. 40-60°C), m.p. 74°C. *Anal.* for $C_{17}H_{23}NO_3$: C,H,N.

N-[2-(3,4-Dimethoxyphenyl)ethyl]-2-pyridylacetamide

Homoveratrylamine (11.9 g ; 0.066 mole) and methyl 2-pyridylacetate (10 g ; 0.066 mole) were heated together in an open vessel for 4 h. The dark oil was cooled and stirred with ether (100 ml). The pale yellow solid (14.5 g ; 74 %), m.p. 98-99°C, was filtered, dried, and crystallised from *n*-hexane — ethyl acetate to give the *amide* as fine needles, m.p. 99.5-100.5°C [Lit (26) 94-95°C].

NN-Bis-[2-(3,4 - dimethoxyphenyl)ethyl]alkane - αω-dicarboxamides **12**

The dicarboxylic acid diethyl esters (1 mole) were heated with homoveratrylamine (2.05 mole) at 180-190°C for 3 h. The crystalline masses obtained on cooling crystallised from ethanol or aqueous ethanol as colourless needles. Relevant data are presented in Table III.

TABLE III

Compound	m.p. (°C)	% Yield	Lit (27) m.p.
12a	162 - 163	56	169
12b	160	40	161
12c	155 - 156	41	156
12d*	152 - 153	81	155 - 156

* Synthesised from decane-1,10-dicarboxylic *acid*.

αω-Bis-(3,4-dihydro-6,7-dimethoxyisoquinolin-1-yl)alkanes **13**

The appropriate bis-amide (**12**, *ca* 7.0 g) in dry toluene (60 ml) was stirred under reflux with phosphoryl chloride (30 ml) for 1 h. The liquid was decanted from the solid which precipitated on cooling. The solid, after washing with petroleum ether (b.p. 40-60°C) and drying, was suspended in ethanol (25 ml), and basified with sodium hydroxide solution (5N). The partially crystallised solid obtained on addition of water (*ca* 200 ml) was extracted with chloroform (2 × 100 ml). The combined extracts were washed with water, dried (Na_2SO_4), and evaporated to leave a yellow oil which solidified on scratching. The percentage yields, crystallising solvents, and m.p.'s for the compounds are presented in Table IV.

1,2,3,4-Tetrahydro-6,7-dimethoxy-1-(2-pyridylmethyl)isoquinoline

N-[2-(3,4-Dimethoxyphenyl)ethyl]-2-pyridylacetamide (14.0 g ; 0.047 mole) in dry toluene (60 ml) was refluxed with phosphoryl chloride (40 ml) for 3 h. The partially cooled reaction mixture was poured into light petroleum (b.p. 60-80°C ; 400 ml) and the liquid was decanted from the semi-solid which precipitated. The precipitate was washed well with light petroleum (b.p. 40-60°C), dried, and crystallised from ethanol-ether to give 3,4-dihydro-6,7-

TABLE IV

Compound No.	% Yield from Amide, 12	Crystallising solvent	m.p. (°C)	Lit. m.p	Ref.
13a	88	Ethyl acetate	171-172	172-173	27
13b	87	Hexane ethyl acetate	102	92	28
13c	92	Hexane ethyl acetate	115	116	27
13d	94	Hexane ethyl acetate	120-121	117-118	29

dimethoxy-1-(2-pyridylmethyl)isoquinoline dihydrochloride, m.p. 203°C (d) [Lit (30) 207°C (d)].

The above salt, in ethanol (60 ml; 95 %), was treated with sodium borohydride (4.0 g; 0.11 mole) in portions over 1 h and the mixture stirred for 18 h. The excess borohydride was destroyed by the cautious addition of dilute hydrochloric acid and the mixture evaporated to dryness *in vacuo*. The off-white crystalline mass remaining was suspended in water (200 ml), basified with sodium hydroxide solution (5N), and the liberated amine extracted with ether (2 × 200 ml). The combined extracts were washed with water, dried (Na_2SO_4), filtered, and treated with an ethereal hydrogen chloride solution. The solid obtained crystallised from methanol-ether in minute, colourless needles of 1,2,3,4-tetrahydro-6,7-dimethoxy-1-(2-pyridylmethyl)isoquinoline dihydrochloride (8.6 g; 52 %), m.p. 189-190°C (d) [Lit (30) 190-191°C (d)]. The free base was a pale yellow, viscous oil.

1-(Cyclopent-2-enylmethyl)-1,2,3,4-tetrahydro-6,7-dimethoxyisoquinoline

N-[2-(3,4-Dimethoxyphenyl)ethyl]cyclopent-2-enylacetamide (10.0 g; 0.035 mole) treated as described for the preparation of 1,2,3,4-tetrahydro-6,7-dimethoxy-1-(2-pyridylmethyl)isoquinoline (except that oxalic acid solution replaced ethereal hydrogen chloride solution) gave the required *amine oxalate* (7.3 g; 58 %) as colourless, crystalline rosettes from ethanol, m.p. 198°C. *Anal.* for $C_{19}H_{25}NO_6$: C,H,N. The *free base*, obtained by ether extraction, was a colourless oil which partially crystallised on standing.

1,2,3,4-Tetrahydro-6,7-dimethoxyisoquinoline

Methylal (50 g; 0.66 mole) was added to homoveratrylamine (100 g; 0.55 mole) in water (200 ml) and concentrated hydrochloric acid (60 ml), and the mixture warmed on a boiling water bath for 8 h. Removal of solvents *in vacuo* gave a dark crystalline mass which crystallised as needles of 1,2,3,4-tetrahydro-6,7-dimethoxyisoquinoline hydrochloride (110 g; 88 %), m.p. 256-257°C [Lit (31) 253°C].

The free base, obtained by basification of the hydrochloride with sodium hydroxide solution, had m.p. 86°C [Lit (32) 84-85°C].

1,2,3,4-Tetrahydro-6,7-dimethoxy-1-(3,4-dimethoxybenzyl)isoquinoline (tetrahydropapaverine) was prepared as previously described (6).

ω,ω-Bis-(1,2,3,4-tetrahydro-6,7-dimethoxyisoquinolin-1-yl)alkanes **2**

The appropriate dihydroisoquinoline, **13** (*ca* 1.0 g) in ethanol (20 ml; 95 %) was treated cautiously with sodium borohydride (*ca* 0.5 g) over 0.5 h. The mixture was stirred for 2.5 h at room temperature, and the excess borohydride destroyed with dilute hydrochloric acid. Ethanol was removed *in vacuo*, and the aqueous suspension basified with sodium hydroxide solution. The liberated amine was extracted with chloroform (2 × 50 ml), and the combined extracts washed with water, dried (Na_2SO_4), and evaporated to leave the corresponding bis-amine, **2**. All the products were colourless, viscous oils, and yields were 98-100 % of theoretical. Melting point data for the corresponding dihydrochlorides are recorded in Table V. No attempt was made to establish the stereochemical composition (racemate and/or meso form) of the salts.

TABLE V

Compound	Melting point (°C) and Crystallising Solvent	Lit (29) m.p.
2a	257-258 (d) (water)	260-261 (d)
2b	260-263 (d) (methanol)	264-265 (d)
2c	253-255 (d) (aqueous ethanol)	258-259 (d)
2d	227-230 (ethanol)	232-234

Ethylene glycol diacrylate **3**

Acryloyl chloride (31.8 ml; 0.4 mole) in dry benzene (60 ml) was added over 0.5 h to a mechanically stirred mixture of ethylene glycol (12.4 g; 0.2 mole), triethylamine (60 ml; 0.4 mole) and pyrogallol (0.1 g) in dry benzene (100 ml) at 5°C. A further quantity of benzene (100 ml) and triethylamine (10 ml) was added, and the mixture stirred at 50°C for 0.5 h. Filtration and removal of solvent *in vacuo* gave a yellow oil which was distilled. The fraction (17.6 g; 52 %), b.p. 118-121°C/25 mm Hg [Lit (34, 35) b.p. 66-68°C/1-2 mm Hg and 75°C/1 mm Hg respectively], was collected as a colourless oil, using distillation apparatus protected from light.

1,10-Bis-dimethylamino-4,7-dioxa-3,8-dioxodecane

A solution of dimethylamine (3.53 g; 0.078 mole) in dry ether (25 ml) was added during 5 min to a cooled, stirred solution of ethylene glycol diacrylate (5.0 g; 0.029 mole) in dry ether (25 ml). The solution was kept at room temperature for 20 h and then evaporated to leave the bis-amine (7.65; 100 %) as an almost colourless oil.

The dimethiodide, **11a**, was prepared by adding methyl iodide (10 ml) to a solution of the bis-amine (2.0 g; 0.0077 mole) in methanol (20 ml). After 2 h, the mixture was diluted with ether (20 ml) and the precipitate crystallised from methanol-ether to yield colourless needles (3.2 g; 76 %), m.p. 170-175°C [Lit (36) 182°C]. *Anal.* $C_{14}H_{30}I_2N_2O_4$: C,H,N.

1,10-Bis-diethylamino-4,7-dioxa-3,8-dioxodecane

A solution of diethylamine (5 ml; 0.05 mole) in dry ether (5 ml) was added during 5 min to ethylene glycol diacrylate (2.0 g; 0.012 mole) in dry ether (10 ml) at 0°C. The mixture was stirred at room temperature for 18 h, and then the solvent and excess diethylamine were removed *in vacuo*. 1,10-Bis-diethylamino-4,7-dioxa-3,8-dioxodecane (3.7 g; 100 %) was a pale yellow oil which showed a single spot on tlc (Polygram Alox. N for tlc; ethanol:ethyl acetate, 1:1, iodoplatinate spray reagent; 37).

The Dimethiodide, **11b**, was obtained by adding the bis-amine (0.4 g; 0.0013 mole) in acetonitrile (5 ml) dropwise to refluxing methyl iodide (10 ml), and continuing reflux for 2 h. Addition of ether (20 ml) gave an oil which crystallised from ethanol as colourless crystals (0.63 g; 83 %), m.p. 143-144°C. *Anal.* for $C_{16}H_{34}I_2N_2O_4$, $0.5H_2O$: C,H,N.

1,10-Bis-(1,2,3,4-tetrahydro-6,7-dimethoxyisoquinolin-2-yl)-4,7-dioxa-3,8-dioxodecane **6a** *and 1-substituted derivatives* **6b-6d**

The following general method for addition was employed. The appropriate secondary amine (*ca* 5.0 g; 2 mole equivalents) and ethylene glycol diacrylate (1 mole equivalent) in dry benzene (20 ml) were stirred for 48 h under reflux and excluding light. The

solvent was removed *in vacuo* and the residual oil dissolved in chloroform (15 ml). Ether (300 ml) was then added, followed by a saturated solution of oxalic acid dihydrate in ether (300 ml). The precipitated solid was filtered off, washed well with ether, dried *in vacuo* over P_2O_5, and crystallised from ethanol. Yields and melting points for the *bis-amine oxalates*, **6a**, **6b** and **6d**, are presented in Table VI. The *bis-amine oxalate*, **6c**, could not be crystallised, and the crude *amine* was used in the final quaternisation step. *Bisamine*, **6d**, had m.p. 47-49°C (Reickert's microscopic melting point apparatus) and gave a *dihydrochloride*, m.p. 181-184°C. *Anal.* for $C_{48}H_{70}Cl_2N_2O_{18},H_2O$: C,H,N.

TABLE VI

Compound	Dioxalate Salt	
	% Yield	m.p. (°C)
6a	60	202.5-204 (d)
6b	65	78-88
6d	69	124-128

The purified *bis-amines* were liberated by dissolving the oxalate salts in water, basifying with dilute sodium carbonate solution and solvent extraction. The corresponding *methiodide quaternary salts*, **7a**, **7c** and **7e** (Table VII) were prepared by dissolving the amine (*ca* 1.0 g) in chloroform (10 ml) and adding methyl iodide (10 ml). After leaving for 20 h at room temperature, the filtered reaction mixture was added drop by drop to mechanically stirred, filtered, dry ether (*ca* 500 ml). The flocculent solid produced was filtered off, washed copiously with ether and dried. The solid was dissolved in methanol (10 ml) and re-precipitated by adding to mechanically stirred dry ether (500 ml). The allyl compound, **7b**, was prepared similarly, using allyl bromide in place of methyl iodide. Methiodide, **7d**, was prepared using acetonitrile in place of chloroform. The mesylate salt, **7f**, was prepared using freshly distilled methyl methanesulphonate in place of methyl iodide. Date relating to these salts are given in Table VII.

TABLE VII

Compound	X	m.p. (°C)	% Yield	Microanalysis	
7a	I	118-120	81	$C_{32}H_{46}I_2N_2O_8$	C,H,N
7b	Br	96-101	77	$C_{36}H_{50}Br_2N_2O_8,H_2O$	C,H,N
7c	I	133-144	90	$C_{44}H_{62}I_2N_2O_8$	C,H,N
7d	I	Softens at 148	20[1]	$C_{46}H_{62}I_4N_4O_8$	C,H,N
7e	I	120-130 (Softens at 95)	95	$C_{50}H_{66}I_2N_2O_{12}$	C,H,N
7f	CH_3SO_3	100-110	90	$C_{52}H_{72}N_2O_{18}S_2,3H_2O$	C,H,N

[1] Crystallised from acetonitrile — methyl iodide (1:1).

Hofmann elimination — gas chromatographic characterisation of compounds **7e** and **7f**

Reference samples of methyl acrylate, ethyl acrylate, *NN*-dimethylbenzylamine and ethylene glycol diacrylate were applied in standard amounts to a column of 10 % Carbowax 20M on Chromosorb W AW-DMCS (80-100 mesh) in a Perkin-Elmer F11 fitted with a flame-ionisation detector and temperature programmer. The quaternary salts, **7e** and **7f**, dissolved in chloroform (10 mg/ml) were treated with *NN*-dimethylbenzylamine in chloroform to initiate Hofmann elimination, and appropriate aliquots applied to the column. Methyl acrylate and ethyl acrylate applied to the column at 50°C had retention times of 0.75 and 1.1 min respectively. Ethyleneglycol diacrylate and the *NN*-dimethylbenzylamine-treated compounds, **7e** and **7f** at column temperatures of 50°C held for 3 min and raised to 115°C at 20°C per min had identical retention times of 6.6 min.

αω - Bis - (1,2,3,4-tetrahydro-6,7-dimethoxy-2-(2-methoxycarbonylethyl) isoquinolin-1-yl)alkanes **5**

The following general method was adopted. The appropriate αω-bis-(1,2,3,4-tetrahydro-6,7-dimethoxyisoquinolin-1-yl)alkane (**2** ; *ca* 3.0 g) was refluxed with methyl acrylate (10 ml) for 2 h. The cooled reaction mixture was dissolved in ether (*ca* 50 ml), and filtered to remove traces of undissolved solid material. The ethereal solution was washed with dilute hydrochloric acid (1N ; 2 × 30 ml) and the combined aqueous layers re-extracted with ether (50 ml). The aqueous layer was then basified with sodium hydroxide solution (5N), and the liberated amine extracted quickly with ether (2 × 100 ml). The combined extracts were washed with water (2 × 30 ml), dried (Na_2SO_4), and evaporated to leave the corresponding *αω-bis-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-(2-methoxycarbonylethyl)isoquinolin-1-yl)alkane* **5**. Yields were 84-99 % of theoretical. The corresponding *dimethiodides* (**1a-1d** ; Table I), were prepared by the general method described above for the preparation of 1,10-bis-(1,2,3,4-tetrahydro-6,7-dimethoxyisoquinolin-2-yl)-4,7-dioxa-3,8-dioxodecane dimethiodide and 1-substituted derivatives. Data relating to the salts prepared are presented in Table VIII.

TABLE VIII

Compound	m.p. °C	% Yield	Microanalysis	
			Formula	
1a	140-150	81	$C_{36}H_{54}I_2N_2O_8,H_2O$	C,H,N
1b	130-139	93	$C_{38}H_{58}I_2N_2O_8,H_2O$	C,H,N
1c	127-135	84	$C_{40}H_{62}I_2N_2O_8$	C,H,N
1d	99-121	79	$C_{42}H_{66}I_2N_2O_8,0.5H_2O$	C,H,N

Neuromuscular blocking potency

Neuromuscular blocking potencies were determined on cat gastrocnemius muscle-sciatic nerve preparations as previously described (1). The results are given in Tables I and II.

Vagal block

Vagal block was determined in anaesthetized cats by periodic stimulation of the cardiac stump of the cut right cervical vagus nerve (10-20 Hz, 1m sec duration, supramaximal voltage) and measurement of the ensuing bradycardia as a percentage of the initial response.

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RÉSUMÉ

Les auteurs ont préparé deux séries de sels d'ammonium bisquaternaires portant, adjacentes au carbone-β du groupe ammonium, des fonctions ester. Ces composés sont conçus pour subir *in vivo*

une élimination d'Hofmann. Leur activité éventuelle comme curarisants a été évaluée sur le Chat. Les variations de la durée de l'action et de sa puissance ainsi que l'effet de blocage du nerf vague sont rapportés et reliés à la structure.

Zusammenfassung

Zwei Reihen bisquartärer Ammoniumsalze, die am β-Kohlenstoff zur Ammoniumgruppe eine Estercarbonyl-Gruppe tragen, wurden synthetisiert. Diese Verbindungen sollten *in vivo* einer Hofmann-Eliminierung unterliegen und wurden auf ihren curarisierenden Effekt an der Katze getestet. Dauer und Intensität der blockierenden Wirkung auf den Vagus werden mit der Struktur der Verbindungen in Zusammenhang gebracht.

References

(1) J.B. Stenlake, J. Urwin, R.D. Waigh and R. Hughes, *Eur. J. Med. Chem.*, 1979, **14**, 77.
(2) J.B. Stenlake, J. Urwin, R.D. Waigh and R. Hughes, *Eur. J. Med. Chem.*, 1979, **14**, 85.
(3) H.O.J. Collier and E.P. Taylor, *Nature* (*London*), 1949, **164**, 491.
(4) A. Bischler and B. Napieralski, *Ber.*, 1893, **26**, 1903.
(5) W.M. Whaley and T.R. Govindachari, *Organic Reactions*, Ed. R. Adams, Wiley, New York, 1951, **6**, 74.
(6) J.B. Stenlake, W.D. Williams, N.C. Dhar and I.G. Marshall, *Eur. J. Med. Chem.*, 1974, **9**, 233.
(7) E.P. Taylor, *J. Chem. Soc.*, 1952, 142.
(8) A. Pictet and T. Spengler, *Ber.*, 1911, **44**, 2030.
(9) W.M. Whaley and T.R. Govindachari, *Organic Reactions*, Ed. R. Adams, Wiley, New York, 1951, **6**, 151.
(10) G. Kalaus, L. Toke and C. Szantay, *Acta Chim. Hung.*, 1970, **63**, 443.
(11) T. Heou-Feo, *Bull. Soc. chim. Fr.*, 1935, **2**, 103 ; *Chem. Abstr.*, 1935, **29**, 33418.
(12) A. Brossi, L.H. Chopard-dit-Jean, J. Würsch and O. Schnider, *Helv. Chim. Acta*, 1960, **43**, 583.
(13) E.C. du Feu, F.J. McQuillin and R. Robinson, *J. Chem. Soc.*, 1938, 1097.
(14) F.J. McQuillin and R. Robinson, *J. Chem. Soc.*, 1938, 1097.
(15) A. Streitwieser, *Chem. Rev.*, 1956, **56**, 571.
(16) H.C. Brown and N.R. Eldred, *J. Am. Chem. Soc.*, 1949, **71**, 445.
(17) Unpublished observations from these laboratories.
(18) C.M. Smith, E.W. Pelikan, L.R. Maramba and K.R. Unna, *J. Pharmacol. Exp. Ther.*, 1953, **108**, 317.
(19) D. Bovet, F. Bovet-Nitti, S. Guarino, V.G. Longo and M. Marotta, *R.C. Ist. sup. Sanit.*, 1949, **12**, 106.
(20) G.A. Mednikyan and G.G. Gevorkyan, *Biol. Zh. Arm.*, 1969, **22**, 36 ; *Chem. Abstr.*, 1969, **71**, 111205f.
(21) S. Thesleff and K.R. Unna, *J. Pharmacol. Exp. Ther.*, 1954, **111**, 99.
(22) J.M. van Rossum and E.J. Ariëns, *Arch. Int. Pharmacodyn.*, 1959, **118**, 393.
(23) W.C. Bowman, *Progress in Medicinal Chemistry*, Eds. G.P. Ellis and G.B. West, London, Butterworths, 1962, **2**, 88.
(24) P.G. Waser and H. Harbeck, *Anaesthetist*, 1962, **11**, 33.
(25) H.O.J. Collier, J.M.Z. Gladych, B. Macauley and E.P. Taylor, *Nature* (*London*), 1958, **182**, 1424.
(26) F. Zymalkowski and B. Trenktrog, *Arch. Pharm. Berl.*, 1960, **293**, 47 ; *Chem. Abstr.*, 1961, **55**, 21108e.
(27) R. Child and F.L. Pyman, *J. Chem. Soc.*, 1929, 2010.
(28) G. Hahn and H.F. Gudjons, *Ber.*, 1938, **71**, 2183.
(29) L.J. Fliedner, J.M. Schor, M.J. Myers and I.J. Pachter, *J. Med. Chem.*, 1971, **14**, 580.
(30) B. Ymato, K. Mashimo, M. Hirakura, O. Yamagata and S. Kurihara, *Yakugaki Zasshi*, 1967, **87**, 1083 ; *Chem. Abstr.*, 1968, **68**, 104952g.
(31) J.S. Buck, *J. Am. Chem. Soc.*, 1934, **56**, 1769.
(32) J.M. Bobbitt, J. McNewkiely, K.L. Khanna and R. Ebermann, *J. Org. Chem.*, 1965, **30**, 2247.
(33) F.L. Pyman ,*J. Chem. Soc.*, 1909, 1610.
(34) J.G. Burtle, J.C. Saam and L.C. Mokrasch, *J. Org. Chem.*, 1952, **17**, 1302.
(35) F. Henry and H. Jullien, *C.R. Acad. Sci., Paris*, 1972, **274**, 639.
(36) R. Fusco, G. Palazzo, S. Chiavarelli and D. Bovet, *Gazz. Chim. Ital.*, 1949, **79**, 836.
(37) L.R. Goldbaum and L. Lazyak, *Anal. Chem.*, 1956, **28**, 1289.

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Bis-3, 4-Dihydroisoquinolinium salts as potential neuromuscular blocking agents

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A series of *NN*-polymethylene-*bis*-3,4-dihydroisoquinolinium salts has been prepared and examined for neuromuscular blocking activity. Some members of the series have been shown to be potent, neuromuscular blocking agents, but with an unacceptable level of vagal blockade for clinical use.

In the course of studies aimed at producing a clinically useful neuromuscular blocking agent (1, 2), we prepared as an intermediate the bis-quaternary 3,4-dihydroisoquinolinium salt (**1b**). This salt resembles a series of 1,2,3,4-tetrahydroisoquinolinium salts prepared by TAYLOR (3), of which one (Laudexium ; **2**) was used clinically, although it suffered from the disadvantage of having too long a duration of action. More significantly, a series of 1,1-linked *bis*-3,4-dihydroisoquinolinium salts (**3**) has been prepared by COPP *et al* (4) and shown to possess adequate potency and short duration of action in cats. When the intermediate (**1b**) proved to be active and shorter acting (*ca* 14 min) than tubocurarine (*ca* 26 min), it was decided to explore the series further, with the objective of increasing activity and reducing vagal block, since this latter feature had resulted in withdrawal of one of the related 1,1-polymethylene-3,4-dihydroisoquinolinium compounds (**3**) from clinical trial (5).

CHEMISTRY

The bis-quaternary salts, **1a**, **1c-1m**, and **4**, were synthesized by treatment of the corresponding 3,4-dihydroisoquinoline with the appropriate α,ω-dihaloalkane. The required 3,4-dihydroisoquinolines were prepared by BISCHLER-NAPIERALSKI cyclisation (6, 7) of the appropriate amides. In general, these 3,4-dihydroisoquinolines are stable (7). 1-benzyl-3,4-dihydroisoquinolines, however, are exceptional in that they undergo air-oxidation to 1-benzoyl-3,4-dihydroisoquinolines in neutral or alkaline media (7, 8). Consequently, special care was required in the quaternisation of 3,4-dihydropapaverine.

The compound, **5**, was obtained by quaternisation of the 1,4-*bis*-[3,4 - dihydro-6,7-dimethoxyisoquinolin-1-yl)methoxy]benzene with an excess of methyl iodide. The unsymmetrical bis-quaternary compound, **6**, was prepared in two stages, firstly by reaction of 3,4-dihydropapaverine with a large excess of decamthylene di-iodide to give an intermediate monoquaternary, followed by quaternisation of the latter using an excess of 3,4-dihydro-6,7-dimethoxy-1-methylisoquinoline.

PHARMACOLOGICAL RESULTS AND DISCUSSION

Methoxy substituents in the isoquinoline ring enhance neuromuscular blocking potency substantially (3). Accordingly, efforts were concentrated on compounds possessing the readily available 6,7-dimethoxy substitution pattern (Table I). The first compounds investigated possessed the 10-carbon separation of onium centres present in decamethonium and Laudexium (3), and in the related 1,1-linked 3,4-dihydroisoquinolinium salts (4). Potency in cats increased substantially in compounds **1e**, **1i** and **1l** with alkyl substituents in place of hydrogen at C-1 (**1b**) of the dihydroisoquinolinium units, and reached a peak at ethyl in compound **1i**, thereafter decreasing in the propyl compound, **1l**. Increase in inter-onium chain length from 10 to 11 or 12 methylene units in compounds **1f** and **1j**, and in **1g** and **1k** respectively, in general reduced the activity with both methyl and ethyl C-1 substituents, with the exception of **1f** in cats. On the other hand, reduction in chain length to eight methylene units increased activity to a maxmum in **1d** where the C-1 substituent was methyl. The activity in **1h** with ethyl at C-1 was less pronounced. Further reduction of the inter-onium chain to six methylene units, attempted only with a C-1 methyl substituent, resulted in a subtantial drop in activity in compound **1c** to the same level as **1e** and **1f**. It is evident, therefore, that in this series as in many others, both inter-onium distance and the presence of appropriate adjacent lipophilic groups, capable of interacting with a hydrophobic bonding receptor subsite, are important determinants of activity (9). It is of

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interest in this context that super-position of structures **1d** (octamethylene chain ; C-1 = Me) and **1h** (decamethylene chain ; C-1 = Et) as at a bis-anionic receptor, shows near super-position of the C-1 methyl groups of **1d** with the terminal methyls of the C-1 ethyl groups of **1h**, yet potency of **1h** was less than that of **1d**. However, all these compounds also showed powerful and persistent vagal block ; sufficient to render them clinically valueless (Table II).

TABLE I. — *Mean neuromuscular blocking potencies of bis-3,4-dihydroisoquinolinium salts.*

Compound	R	R'	n	X	Relative Molar Neuromuscular Blocking Potency (Tubocurarine = 100)	
					Rat diaphragm	Anaesthetised cats
1a	H	H	10	I	0.1(2)[3]	–
1b	OMe	H	10	I	0.65(2)	5(1)[3]
1c	OMe	Me	6	Bz_4	–	6?(2)
1d	OMe	Me	8	Bz_4	1.20(2)	104(2)
1e	OMe	Me	10	I	2.10(2)	31(3)
1f	OMe	Me	11	Bz_4	1.00(2)	1?(2)
1g	OMe	Me	12	Bz_4	1.00(2)	14(4)
1h	OMe	Et	8	Bz_4	1.30(2)	61(2)
1i	OMe	Et	10	I	1.50(2)	66(3)
1j	OMe	Et	11	Bz_4	1.00(2)	25(5)
1k	OMe	Et	12	Bz_4	1.40(2)	20(3)
1l	OMe	Pr	10	I	1.45(2)	10(3)
1m	OMe	3,4-Dimethoxybenzyl	10	I	1.05(3)	–
5					1.65(2)	50(3)
5					–	0?(2)
6					–	89(2)

[1]. No of animals. [2]. No block recorded at doses up to 4 mg/kg.

TABLE II. — *Mean neuromuscular and vagal blocking doses of bis-3,4-dihydroisoquinolinium salts in cats.*

Compound	PD50[1]	VD50[2]	VD50/PD50
1a	–	–	–
b	2.7 (1)[4]	2.1 (1)	0.78
c	0.39(2)	0.18(2)	0.46
d	0.23(2)	0.20[5](2)	–
e	0.99(2)	0.40[5](2)	–
f	0.41(1)	0.44(1)	1.1
g	2.3 (1)	0.20(3)	0.09
h	0.24(2)	0.24(2)	1.00
i	0.21(3)	0.50[5](3)	–
j	0.62(4)	0.58(4)	0.94
k	1.3 (1)	1.3 (1)	0.87
l	0.72(2)	0.61(2)	0.85
5	0.39(2)	0.65(2)	1.7
5	0[3] (2)	3.2 (2)	–
6	0.19(2)	0.13(2)	0.68

[1] Dose in mg/kg iv producing 50 % neuromuscular block.
[2] Dose in mg/kg ic producing 50 % vagal block.
[3] No block recorded at doses up to 4 mg/kg.
[4] No of animals.
[5] Dose in mg/kg iv producing 100 % vagal block.

Introduction of 3,3-dimethyl substituents into the 3,4-dihydroisoquinolne compounds, **3** (4), increased activity, and did so again in the present series. Thus, **4** was more potent than **1b**, but again vagal block was severe. We, therefore, attempted to modify the basic structure to avoid vagal block, using a 3,4-dimethoxybenzyl substituent at C-1 of the isoquinoline ring in place of alkyl, such a substituent having a beneficial effect in Laudexium ; **2**. Thus, we obtained **1m** (n = 10 ; R = MeO ; R' = 3,4-dimethyoxybenzyl ; X = I) which unfortunately produced an irreversible block on the rat diaphragm.

A number of the 3,4-dihydroisoquinolinium compounds proved to be of relatively short duration of action (*ca* 10-20 min) in cats compared with such compounds as tubocurarine (*ca* 26 min) and Gallamine (*ca* 33 min) (10). It seemed possible that this might be associated with the electron-deficient carbon at C-1, which could be subject to nucleophilic attack by a variety of agents *in vivo*, with removal of the charge on nitrogen (Scheme 1). We, therefore, attempted to combine one of these potentially degradable units with a laudanosine unit in a single bis-quaternary compound **6**, in an endeavour to achieve both short-action and freedom from side-effects. In the event, the product was reasonably potent in cats, but showed severe vagal block.

Scheme 1

Our final attempt to incorporate the 3,4-dihydroisoquinoline unit into an acceptable drug molecule is represented in **5**, which possesses a resemblance to tubocurarine and Laudexium. Surprisingly for a bis-quaternary, this compound had no measurable neuromuscular blocking activity. This lack of blocking effect could be associated with the proximity of the ether oxygen atoms to the onium centres (11).

EXPERIMENTAL

Unless stated otherwise, melting point were recorded on a Kofler Heizbank 184321 melting point apparatus. Infrared spectra were recorded on a Perkin Elmer 157 instrument using liquid films or KCl discs (for solids). Proton magnetic resonance spectra were recorded on a Perkin Elmer R12 instrument operating at 60 MHz. TMS was used as internal standard. Microanalytical results (C, H, N except where otherwise stated) were within ± 0.4 % of theoretical.

N-[2-(3,4-Dimethoxyphenyl)ethyl]propanamide

Propanoic anhydride (30 ml ; 0.23 mole) was added cautiously to homoveratrylamine (20 g ; 0.11 mole) at 5°C and the mixture allowed to stand for 10 min. Ethanol (15 ml) was added and the mixture boiled for 5 min, cooled, diluted with water (200 ml) and extracted with chloroform (100 ml ; 50 ml). The combined extracts were washed with water, dried (Na_2SO_4) and evaporated to leave a yellow oil which solidified on trituration with several quantities of ether — light petroleum (b.p. 40-60°). Crystallisation from ethyl acetate — light petroleum (60-80°) gave *N*-[2-(3,4-dimethoxyphenyl)ethyl]propanamide (14 g ; 53 %), m.p. 57-59°C [Lit. (12) 57.5-59°C].

N-[2-(3,4-Dimethoxyphenyl)ethyl]butanamide

Treatment of homoveratrylamine (20 g ; 0.11 mole) with butanoic anhydride (30 ml ; 0.19 mole) by the method described for the preparation of *N*-[2-(3,4-dimethoxyphenyl)ethyl]propanamide, gave *N*-[2-(3,4-dimethoxyphenyl)ethyl]butanamide (14.5 g ; 50 %) as colourless needles from ethyl acetate — light petroleum (b.p. 40-60°C), m.p. 47-50°C [Lit. (12) 51-53°C].

N-[2-(3,4-Dimethoxyphenyl)ethyl]-3,4-dimethoxyphenylacetamide

Homoveratrylamine (1.025 mole) was heated with 3,4-dimethoxyphenylacetic acid (1 mole) in an open vessel at 190-210°C for 2 h. The solid mass obtained on cooling crystallised from ethanol as colourless needles (92 %), m.p. 118-120°C [Lit. (13) 124°C].

NN'-Bis-[2-(3,4-dimethoxyphenyl)ethyl]hydroquinone-OO'-diacetamide

Homoveratrylamine (10 g ; 0.055 mole) and hydroquinone-*OO'*-diacetic acid (5 g ; 0.022 mole) were heated together in an open vessel for 2 h at 190-210°C. The solid mass obtained on cooling crystallised from ethanol as fine, colourless needles which were filtered off, washed well with ethanol and dried. The bis-amide (9.5 g ; 75 % had m.p. 138-139°C [Lit. (14) 143-145°C]. ν_{max} 3300, 1650, 1510 cm^{-1}. δ ($CDCl_3$) : 2.80 (4H, t, 2×ArCH_2-), 3.57 (4H, t, 2×CH_2NHCO-), 3.85 (12H, s, 4×ArOCH_3), 4.42 (4H, s, 2×ArOCH_2CO-), 6.60-6.90 (12H, m, Ar-*H* + 2×N*H*).

3,4-Dihydroisoquinoline, *3,4-dihydro-6,7-dimethoxyisoquinoline* and *3,4-Dihydro-6,7-dimethoxy-1-methylisoquinoline* were prepared as described previously (1).

1-Ethyl-3,4-dihydro-6,7-dimethoxyisoquinoline

N-[2-(3,4-dimethoxyphenyl)ethyl]propanamide (5 g ; 0.021 mole) and polyphosphoric ester (25 g) were heated together at 120°C for 1 h. The mixture was cooled, diluted with water (100 ml), and basified with sodium hydroxide solution (5N ; 100 ml). The aqueous solution was extracted with chloroform (100 ml ; 50 ml), and the combined extracts were washed with water, dried (Na_2SO_4) and evaporated to give an oil (4.1 g ; 89 %) sufficiently pure for subsequent use. Pure 1-ethyl-3,4-dihydro-6,7-dimethoxyisoquinoline had b.p. 106-110°C/0.05 mm Hg [Lit. (12) 127-130°C/0.3 mm Hg].

3,4-Dihydro-6,7-dimethoxy-1-propylisoquinoline

N-[2-(3,4-Dimethoxyphenyl)ethyl]butanamide (5 g ; 0.019 mole), treated as described in the synthesis of 1-ethyl-3,4-dihydro-6,7-dimethoxyisoquinoline, gave the product as an oil (4.2 g ; 90 %) sufficiently pure for subsequent use. Pure 3,4-dihydro-6,7-dimethoxy-1-propylisoquinoline had b.p. 109-110°C/0.05 mm Hg [Lit. (12) 150-170°C/2-3 mm Hg].

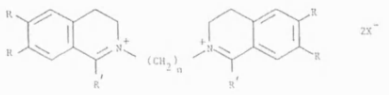
3,4-Dihydro-6,7-dimethoxy-1-(3,4-dimethoxybenzyl)isoquinoline-(3,4-dihydropapaverine)

N-[2-(3,4-Dimethoxyphenyl)ethyl]-3,4-dimethoxyphenylacetamide (0.024 mole) was refluxed in dry toluene (60 ml) with phosphoryl chloride (40 ml) for 3 h. The partially cooled reaction mixture was poured into light petroleum (b.p. 60-70°C ; 40 ml), and the liquid decanted from the semi-solid precipitate over 15 min. The semi-solid was washed well with light petroleum (b.p. 40-60°C), dried, and crystallised from ethanol-ether to give 3,4-dihydropapaverine hydrochloride. The free base was obtained by basification and rapid extraction in the usual way immediately prior to use.

1,4-Bis[(3,4-dihydro-6,7-dimethoxyisoquinolin-1-yl)methoxy]benzene

NN-Bis-[2-(3,4-dimethoxyphenyl)ethyl]hydroquinone-*OO'*-diacetamide (5 g ; 0.0064 mole) in dry toluene (60 ml) was stirred under reflux with phosphorus oxychloride (30 ml ; 0.33 mole) for 1 h. The liquid was decanted from the solid which precipitated on cooling. The solid, after washing with light petroleum (40-60°C) and drying, was suspended in ethanol (25 ml) and basified with sodium hydroxide solution (5N). The partially crystalline solid obtained on addition of water (200 ml) was extracted with chloroform (2 × 100 ml). The combined extracts were washed with water, dried (Na_2SO_4), and evaporated to leave a yellow solid. Crystallisation from ethanol gave the required product (3.8 g ; 80 %), m.p. 174°C [Lit. (14) 166-168°C] as long, colourless needles. ν_{max} : 1640, 1600, 1500 cm^{-1}. δ ($CDCl_3$) : 2.63 (4H, t, 2×ArCH_2-), 3.78 (4H, t-, CH_2N=C-), 3.84 (6H, s, 2×ArOCH_3), 3.92 (6H, s, 2×ArOCH_3), 5.05 (4H, s, 2×ArOCH_2-C=N-), 6.71 (2H, s, Ar-*H*), 6.98 (4H, s, Ar-*H*), 7.25 (2H, s, Ar-*H*).

The *Dimethiodide* (5) prepared by refluxing the bis-amine with excess methyl iodide for 4 h, had m.p. 228-230°C, from aqueous methanol. Re-crystallisation from aqueous methanol gave the analytical sample, m.p. 229-231°C. ν_{max} : 2930, 2810, 1630, 1605, 1570, 1505 cm^{-1}. *Anal.* for $C_{37}H_{48}I_2N_2O_6$, 1.5 H_2O : C, H, N.



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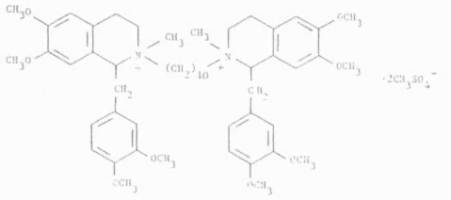

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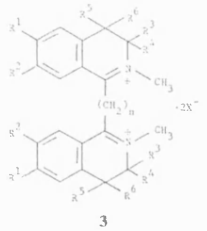

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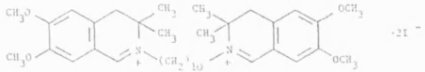

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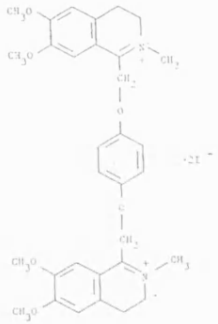

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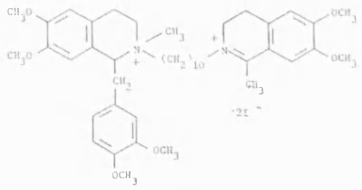

6

6,7-Dimethoxy-1-(3,4-dimethoxybenzyl)-2-methylisoquinoline (laudanosine)

This compound was prepared from tetrahydropapaverine by the Eschweiler-Clarke method (15) and had m.p. 115-116°C.

Bis-*3,4-Dihydroisoquinolinium salts* (1)

The following general method was adopted : the appropriate 3,4-dihydroisoquinoline base (4 mole equivalents ; *ca* 4 g) and alkane-αω-dihalide (1 mole equivalent) in dry benzene (25 ml) were refluxed for about 24 h. The mixture was cooled, ether (*ca* 100 ml) added, and the solid material filtered off, washed well with ether, dried, and crystallised from ethanol. Relevant data for all compounds prepared by this method are presented in Table III.

TABLE III

NN-Polymethylene-bis-3,4-dihydroisoquinolinium salts, 1.

Compound	m.p. (°C)	Formula (Anal. C,H,N)
1a	186 - 188	$C_{28}H_{38}I_2N_2$
c	241 (d)	$C_{30}H_{42}Br_2N_2O_4 . 1\ H_2O$
d	110 - 120	$C_{32}H_{46}Br_2N_2O_4$
e	130 - 140	$C_{34}H_{50}I_2N_2O_4$
f	172	$C_{35}H_{52}Br_2N_2O_4 . 1\ H_2O$
g	138 - 140	$C_{36}H_{54}Br_2N_2O_4 . 1\ H_2O$
h	222 - 224	$C_{34}H_{50}Br_2N_2O_4 . 2\ H_2O$
i	169 - 171	$C_{36}H_{54}I_2N_2O_4$
j	190 - 198	$C_{37}H_{56}Br_2N_2O_4 . 1.5\ H_2O$
k	200 - 203	$C_{38}H_{58}Br_2N_2O_4 . 1\ H_2O$
l	173 - 176	$C_{38}H_{58}I_2N_2O_4$
m	123 - 128	$C_{50}H_{66}I_2N_2O_4$

*1,10-*Bis-*(3,4-Dihydro-6,7-dimethoxy-3,3-dimethyl-2-isoquinolinium) decane di-iodide* (4)

3,4-Dihydro-6,7-dimethoxy-3,3-dimethylisoquinoline (1,2 g ; 0.0055 mole) prepared as described by Copp (16) and decamethylene di-iodide (0.7 g ; 0.0018 mole) in benzene (10 ml) were refluxed for 24 h. Addition of ether (30 ml) to the cooled reaction mixture gave a yellow solid which was filtered off, washed well with ether and dried. Crystallisation from *n*-propanol gave 4 (0.72 g ; 49 %) as yellow prisms, m.p. 170-172°C (softens at 160°C). *Anal.* for $C_{36}H_{58}I_2N_2O_4$; : C, H, N.

1-[1,2,3,4-Tetrahydro-6,7-dimethoxy-1-(3,4-dimethoxybenzyl)-2-methyl-2-isoquinolinium]10-(3,4-dihydro-6,7-dimethoxy-1-methyl-2-isoquinolinium)decane di-iodide (6)

Laudanosine (0.5 g ; 0.0014 mole) and decamethylene di-iodide (5.5 g ; 0.014 mole) in dry benzene (15 ml) were refluxed for 30 h, left at room temperature for 60 h, and then refluxed for a further 5 h. The semi-solid (0.75 g ; 71 %) obtained on careful addition of dry ether to the partially cooled reaction mixture was hygroscopic. A portion of the semi-solid (0.3 g ; 0.0004 mole) in absolute ethanol (5 ml) was refluxed with 3,4-dihydro-6,7-dimethoxy-1-methylisoquinoline (0.5 g ; 0.0024 mole) for 24 h. Addition of ether to the cooled reaction mixture gave a semi-solid which was dissolved in methanol (10 ml) and added dropwise, with filtration, to mechanically stirred, filtered, dry ether (500 ml). Repetition of the precipitation process gave 6 (0.28 g ; 47 %), m.p. 131-136°C with preliminary softening at 124°C. ν_{max} : 2940, 1625, 1510 cm^{-1}. *Anal.* for $C_{41}H_{62}I_2N_2O_6$: C, H, N.

Neuromuscular blocking potency

Neuromuscular blocking potencies were determined on the rat phrenic-nerve diaphragm and cat gastrocnemius muscle-sciatic nerve preparations as previously described (1). The results are given in Table I.

Vagal block

Vagal block was determined in anaesthetised cats by periodic stimulation of the cardiac stump of the cut right cervical vagus nerve (10-20 Hz, 1 m sec duration, supramaximal voltage) and measurement of the ensuing bradycardia as a percentage of the initial response. The results are given in Table II.

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RÉSUMÉ

Préparation d'une série de sels de N,N-polyméthylène-bis-dihydro-3,4 isoquinolinium et étude de leur activité curarisante ; certains dérivés sont de puissants agents de relâchement musculaire de courte durée d'action. Toutefois, le niveau élevé de leur action sur le nerf vague les rend impropres à l'usage clinique.

ZUSAMMENFASSUNG

Eine Reihe von N,N'-Polymethylen-3,4-dihydroisochinolinium-Salzen wurde hergestellt und auf ihre neuromuskuläre Hemmwirkung untersucht. Einige Glieder der Reihe erwiesen sich als starke, kurzfristig wirkende Muskel-relaxantien, hatten aber eine für die klinische Anwendung unannehmbar starke Wirkung auf den Vagus.

REFERENCES

(1) J.B. STENLAKE, J. URWIN, R.D. WAIGH and R. HUGHES, *Eur. J. Med. Chem.*, 1979, **14**, 77.
(2) J.B. STENLAKE, J. URWIN, R.D. WAIGH and R. HUGHES, *Eur. J. Med. Chem.*, 1979, **14**, 85.
(3) E.P. TAYLOR, *J. Chem. Soc.*, 1952, 142.
(4) F.C. COPP, G.G. COKER, A.F. GREEN, R. HUGHES and R.M. NIMMO-SMITH, *Experientia*, 1972, **28**, 47.
(5) R. HUGHES, *Brit. J. Anaesth.*, 1972, **44**, 27.
(6) B. BISCHLER and B. NAPIERALSKI, *Ber.*, 1893, **26**, 1903.
(7) W.M. WHALEY and T.R. GOVINDACHARI, *Organic Reactions*, 1951, **6**, 74.
(8) R.W. ALDER, *Chem. Ind.*, 1973, 983.
(9) J.B. STENLAKE, *Neuromuscular Blocking Agents*, in : Alfred Burger's Medicinal Chemistry, Ed. M.E. Wolff, Wiley-Interscience, New York, 4th edn., Part III, Chapter 46, 1981.
(10) R. HUGHES and D.J. CHAPPLE, *Brit. J. Anaesth.*, 1976, **48**, 59.
(11) J.B. STENLAKE, in : *Progress in Medicinal Chemistry*, Vol. 3, Eds G.P. Ellis and G.B. West, Buttherworths. London, 1963, 1.
(12) P.N. CRAIG, F.P. NABENHAUER, P.M. WILLIAMS, E. MACKO and J. TONER, *J. Amer. Chem. Soc.*, 1952, **74**, 1316.
(13) J.S. BUCK, R.D. HAWORTH and W.H. PERKIN, *J. Chem. Soc.*, 1924, 2176.
(14) M. ONDA, *J. Pharm. Soc.* (Japan), 1954, **74**, 925 ; *Chem. Abstr.*, 1955, **49**, 10962b.
(15) R.N. ICKE, B.B. WISEGARNER and G.A. ALLES, *Organic Syntheses Coll.*, 1955, **3**, 723.
(16) F.C. COPP, Brit. Patent 1,125,619 (1968) ; *Chem. Abstr.*, 1969, **71**, 13033d.

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Biodegradable neuromuscular blocking agents.

Part 4. Atracurium Besylate and related polyalkylene di-esters

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A series of αω-bisbenzylisoquinolinium polyalkylene di-esters designed to undergo Hofmann elimination at physiological pH has been synthesised. Initially, neuromuscular blocking potency increases with interonium spacing up to 13 to 14 atoms, thereafter declining. The most active compounds have been shown to possess good non-depolarising neuromuscular blocking activity with high specificity for the neuromuscular junction and medium duration of action. Atracurium Besylate (1,1',2,2',3,3',4,4'-octahydro-6,6',7,7'-tetramethoxy-2,2'-dimethyl-1,1'-diveratryl-2,2'-(3-11-dioxo-4,10-dioxatridecamethylene)diisoquinolinium dibenzenesulphonate) which is being developed for clinical use as a neuromuscular blocking agent appears to be inactivated by both enzymic and non-enzymic mechanisms. There is evidence to suggest that the chemically-based Hofmann elimination pathway plays an important part in this inactivation.

Previous papers in this series have described the development of a novel approach to biodegradable neuromuscular blocking agents based on Hofmann elimination of suitably substituted quaternary ammonium salts (1-3). In this paper, we describe a continuation of the basic studies which led to the promising derivative of tetrahydropapaverine, the bisquaternary **1a** (Scheme 2) (3). In cats, this compound showed a moderate potency as a non-depolarising neuromuscular blocking agent, with a medium duration of action and with distinct separation between neuromuscular and vagal blockade. Compound **1a** is a reversed ester analogue of a compound, **2**, prepared by COLLIER, GLADYCH, MACAULAY and TAYLOR (4) as a non-depolarising analogue of succinyldicholine. This latter compound, **2**, possessed only one-third of the potency of (+)-tubocurarine and was slightly longer acting (4), whereas **1a** was somewhat shorter acting (recovery from onset of full block *ca* 14-20 min) than (+)-tubocurarine (*ca* 20-33 min), although no more potent than **2**. It would appear, therefore, that **2** is not a good substrate for esterases at the neuromuscular junction which might inactivate it, whereas we have shown that compounds related to **1a**, e.g. **1k**, can also be degraded *in vitro* by a base-catalysed mechanism independent of esterases at physiological pH. We describe here our successful attempts to increase the potency without losing the specificity for the neuromuscular junction which was an attractive feature of **1a**.

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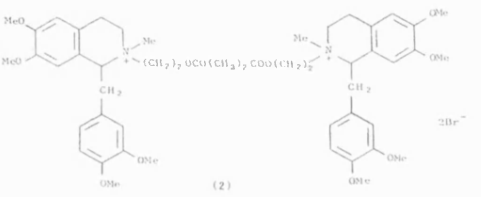
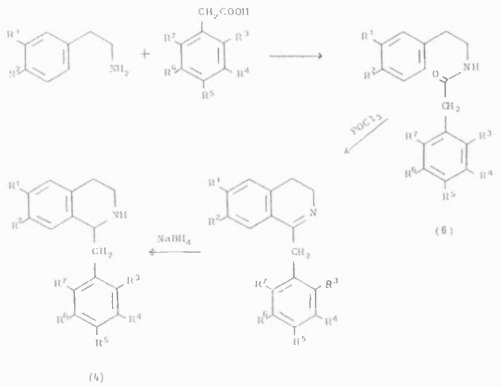


(2)

CHEMISTRY

The bis-quaternary ammonium salts, **1a-1s**, were prepared by the route outlined in Schemes 1 and 2. These compounds are quaternary derivatives of the bis-tertiary amino esters, **3**, obtained by Michael condensation of the 1-benzyl-1,2,3,4-tetrahydroisoquinolines, **4**, with diol diacrylate esters, **5** (5).

The 1-benzyl-1,2,3,4-tetrahydroisoquinolines, **4**, were prepared by Bischler-Napieralski cyclisation (6, 7) of appropriate amides, **6** (Scheme 1), using phosphorus oxychloride, to give the 3,4-dihydroisoquinolines, followed by reduction with sodium borohydride. Due to the tendency of 1-benzyl-3,4-dihydroisoquinolines to undergo air-oxidation to 1-benzoyl-3,4-dihydroisoquinolines (7, 8) in neutral or alkaline media, no attempt was made to isolate the free bases. Since such oxidation does not occur in acidic media (8), this complication was avoided by reducing directly (9) the partially purified 3,4-dihydroisoquinoline proton salt from phosphorus oxychloride cyclisation, and purifying the resulting 1,2,3,4-tetrahydroisoquinoline. 1,2,3,4-tetrahydro-6,7-dimethoxy-1-[2(3,4-dimethoxyphenyl)ethyl]isoquinoline was prepared similarly.



(4)

	R^1	R^2	R^3	R^4	R^5	R^6	R^7
(a)	OMe	OMe	H	H	H	H	H
(b)	OMe	OMe	H	H	OMe	H	H
(c)	OMe	OMe	H	OMe	OMe	H	H
(d)	OMe	OMe	OMe	H	H	OMe	H
(e)	OMe	OMe	H	O—CH_2—O		H	H
(f)	OMe	OMe	H	OMe	OMe	OMe	H
(g)	OMe	OMe	Br	H	OMe	OMe	H
(h)	OMe	OMe	H	Cl	Cl	H	H
(i)	O—CH_2—O		H	OMe	OMe	H	H

Scheme 1

The bis-acrylate esters, **5**, were prepared by the reaction of acryloyl chloride with the appropriate diol in the presence of triethylamine as acid scavenger. All of the liquid acrylates tended to polymerise at elevated temperatures (> *ca* 150°C). Consequently, distillations were performed with the exclusion of light, in the presence of a polymerisation inhibitor such as 4-methoxyphenol, and using the highest possible vacuum. Despite a wide patent literature, the physical constants of most of these diacrylates have only recently been reported (10).

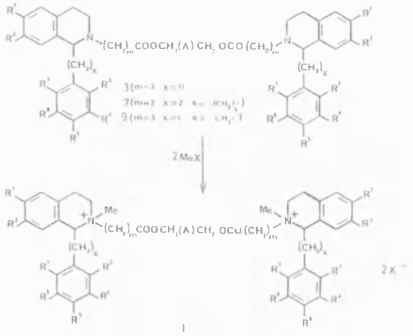
$CH_2{=}CHCOOCH_2$ — (A) — $CH_2OCOCH{=}CH_2$

	A
5a	$-CH_2-$
b	$-(CH_2)_2-$
c	$-(CH_2)_3-$
d	$-(CH_2)_4-$
e	$-(CH_2)_5-$
f	$-(CH_2)_6-$
g	$-CH_2OCH_2-$
h	$-CH_2CHCH_2-$ (with CH_3)
i	

Michael addition of the various 1-benzyltetrahydroisoquinolines, **4**, to appropriate diacrylates, **5**, proceeded smoothly without side-reactions to yield bis-amines, **3** and **7**. Purification of all bis-amines was achieved by crystallisation of dioxalate proton salts. No attempt was made in the present work to establish the racemate — *meso* ratio of these purified oxalates, arising from the asymmetry associated with position 1 of the tetrahydroisoquinoline ring.

The bis-amine, **9**, was prepared by an alternative route. γ-Butyrolactone was treated with propane-1,3-diol in the presence of hydrogen bromide to yield propane-1,3-bis (4-bromobutanoate), and the latter then condensed with tetrahydropapaverine.

The bis-quaternary salts, **1a-1e** and **1k-1q**, were prepared from the corresponding bis-amines by alkylation with methyl iodide. It became evident, however, in the course of preliminary pharmacological studies that the methiodides of this series were not sufficiently soluble to permit the preparation of small volume intravenous injec-



Compound	R^1	R^2	R^3	R^4	R^5	R^6	R^7	m	x	A	Salt (X^-)
1a	OMe	OMe	H	OMe	OMe	H	H	2	1	–	Iodide
											Mesylate
b	OMe	OMe	H	H	H	H	H	2	1	–	Iodide
c	OMe	OMe	H	H	OMe	H	H	2	1	–	Iodide
d	OMe	OMe	OMe	H	H	OMe	H	2	1	–	Mesylate
e	OMe	OMe	H	O-CH_2-O		H	H	2	1	–	Mesylate
f	OMe	OMe	H	OMe	OMe	OMe	H	2	1	–	Mesylate
g	OMe	OMe	Br	H	OMe	OMe	H	2	1	–	Mesylate
h	OMe	OMe	H	Cl	Cl	H	H	2	1	–	Mesylate
i	OMe	OMe	H	OMe	OMe	H	H	2	1	$-CH_2-$	Mesylate
j	OMe	OMe	H	OMe	OMe	H	H	2	1	$-(CH_2)_4-$	Mesylate
k	OMe	OMe	H	OMe	OMe	H	H	2	1	$-(CH_2)_6-$	Iodide
											Mesylate
											Besylate
											Tosylate
											1-Napsylate
											2-Napsylate
l	OMe	OMe	H	OMe	OMe	H	H	2	1	$-(CH_2)_8-$	Iodide
											Mesylate
m	OMe	OMe	H	OMe	OMe	H	H	2	1	$-(CH_2)_{10}-$	Iodide
n	OMe	OMe	H	OMe	OMe	H	H	2	1	$-(CH_2)_{12}-$	Iodide
o	O-CH_2-O		H	OMe	OMe	H	H	2	1	–	Iodide
p	O-CH_2-O		H	OMe	OMe	H	H	2	1	$-(CH_2)_6-$	Iodide
q	OMe	OMe	H	OMe	OMe	H	H	2	1	$-CH_2-O-CH_2-$	Iodide
r	OMe	OMe	H	OMe	OMe	H	H	2	1	$p-CH_2-C_6H_4-CH_2-$	Mesylate
s	OMe	OMe	H	OMe	OMe	H	H	2	1	$-CH_2-CH(CH_3)-CH_2-$	Mesylate
8	OMe	OMe	H	OMe	OMe	H	H	2	2	$-(CH_2)_6-$	Mesylate
10	OMe	OMe	H	OMe	OMe	H	H	3	1	$-(CH_2)_7-$	Mesylate

Scheme 2

tion solutions for clinical use. Attention was turned, therefore, to other alkylating agents, notably methyl methanesulphonate. Careful purification and rigorous exclusion of water were found to be necessary with this reagent, not only to prevent hydrolysis of the quaternary products themselves, but also to avoid hydrolysis of methyl methanesulphonate to methanesulphonic acid. The presence of this acid in the mixture would obviously protonate, rather than quaternise, the tertiary amine, and the use of scrupulously dry precursor bis-amine was found to be essential. This was best obtained by extracting the base, liberated from purified oxalate, with toluene, evaporating to azeotrope water, and completing the process by drying *in vacuo* over P_2O_5 at *ca* 60-80°C. The water-soluble methanesulphonates, **1a**, **1d-1l**, **1r**, **1s**, **8** and **10**, were prepared by this method.

Concern over the stability of **1k** mesylate led to the evaluation of other salts prior to clinical development. The benzenesulphonate (besylate), toluenesulphonate (tosylate) and 1- and 2-naphthalenesulphonates of **1k** were prepared by the same method as the mesylate. Both naphthalenesulphonates had lower solubilities than the methiodide, but the besylate (**1k**,X^- = Ph . SO_3O^-) and tosylate were readily soluble in water to the extent of about 60 and 35 mg/ml at 25°C respectively.

There are four asymmetric centres in all the compounds described, but considerations of symmetry reduce the number of possible isomers to ten. We have evidence that the synthetic methods used give rise to batchwise consistent isomer ratios, and details of these stereochemical studies will be reported in a later paper in the series. The results reported in Table I are for unresolved isomer mixtures.

PHARMACOLOGICAL RESULTS AND DISCUSSION

Neuromuscular blocking potency and the extent of vagal block were measured in anaesthetised cats and Rhesus monkeys. The results are given in Tables I and II.

Consideration of structure **1a** (Scheme 2) suggested three obvious points for modification to enhance potency; alteration of (a) the central chain, (b) the isoquinoline benzene ring, or (c) the 1-benzyl substituent. Alteration of the *N*-methyl substituent would be counter-productive (3) and the two-carbon separation of carbonyl and nitrogen had to be maintained in order to preserve the potential for Hofmann elimination (1-3).

Modification of the 1-benzyl substituents was readily effected by the use of appropriately substituted phenylacetic acids in the synthesis. Choice of substituents was based partly on the usual considerations of bulk, electron distribution and lipophilicity, and partly on availability of suitable phenylacetic acids. The results presented in Tables I and II for the products, **1b-1h**, in each case show a reduction in neuromuscular blocking potency and an increase in effect on the vagus compared to **1a**.

In view of the foregoing results, the 3,4-dimethoxybenzyl group was retained in all but three of the remaining compounds, **1i-1s**. One 3,4-dimethoxyphenethyl homologue, **8**, and two 6, 7-methylenedioxy compounds, **1o** and **1p**, were also prepared, but none of these compounds showed advantage over the corresponding 1-(3, 4-dimethoxybenzyl) compound, **1k**. These results were not encouraging, and left only the central chain of the three parameters described for further modification.

TABLE I. — *Mean neuromuscular blocking potencies and duration of action of ω,ω-bisbenzyltetrahydroisoquinolinium polyalkylene di-esters,* **1**, **8** *and* **10**, *in cats and rhesus monkeys.*

Compound No.	Relative molar potency (Tubocurarine = 100)		Full recovery from onset of complete paralysis (min)	
	Cat	Monkey	Cat	Monkey
1a	58(2)[1]	15(2)[1]	17	13
b	10(2)	–	13	–
c	18(2)	–	14	–
d	11(2)	–	22	–
e	20(2)	–	20	–
f	12(3)	–	20	–
g	15(2)	–	22	–
h	24(2)	–	–	–
i	68(2)	26(2)	24	26
j	133(2)	69(1)	24	25
k	157(6)	157(7)	22 ± 2.9[2]	31.5 ± 2.7[2]
l	228(2)	214(2)	22	25 ± 5.0[2]
m	127(2)	–	24	–
n	121(2)	–	19	–
o	46(2)	–	10	–
p	123(2)	–	27	18
q	39(1)	–	20	–
r	52(2)	–	17	–
s	91(3)	–	35 ± 11.8[2]	–
8	88(3)	–	31 ± 3.8[2]	–
10	111(6)	48(4)	51 ± 5.2[2]	60 ± 33.5[2]

[1] No. of animals.
[2] SEM.

TABLE II. — *Mean neuromuscular and vagal blocking doses of ω,ω-bisbenzyltetrahydroisoquinolinium polyalkylene di-esters* **1**, **8** *and* **10** *in cats and rhesus monkeys.*

Compound	CAT			MONKEY		
	PD50[1]	VD50[2]	VD50/ PD50	PD50[1]	VD50[2]	VD50/ PD50
1a	0.36(2)[3]	2.6	7.2	1.3(2)	6.8	5.2
b	1.2 (2)	1.7	1.0			
c	1.0 (2)	4.7	4.7			
d	1.9 (2)	2.1	1.3			
e	1.0 (2)	1.7	1.7			
f	1.7 (2)	4.5	2.7			
g	1.6 (2)	2.3	1.4			
h	0.7 (2)	3.5	0.4			
i	0.26(2)	7.3	2V	0.75(2)	4.3	5.8
j	0.13(2)	5.4	42	0.27(3)	2.6	9.6
k	0.13 ± 0.01 (6)	3.08 ± 0.76	24.4 ± 6.3	0.12 ± 0.02 (7)	4.70 ± 0.99	39.0 ± 8.4
l	0.09 ± 0.01 (4)	3.02 ± 0.34	34.6 ± 5.5	0.11 ± 0.01 (4)	3.65 ± 0.64	32.6 ± 10.3
m	0.15(2)	2.6	17			
n	0.18(2)	1.5	8.6			
o	0.43(2)	3.1	7.2			
p	0.44 ± 0.16 (4)	2.4 ± 0.5	7.3 ± 2.3	0.47(2)	2.6	5.5
q	0.46(2)	10.8	23			
r	0.49(2)	2.4	4.9			
s	0.28(3)	2.7	9.6			
8	0.24(2)	1.4	5.8			
10	0.16 ± 0.02(6)	2.78 ± 0.44	18.8 ± 3.9	0.41 ± 0.08 (4)	2.61 ± 0.91	6.95 ± 2.94
Tubocurarine	0.13 ± 0.01(4)	0.19 ± 0.01	1.5 ± 0.15	0.12 ± 0.02 (4)	0.64 ± 0.08	6.2 ± 0.98
Dimethylthebaconine	0.02 ± 0.003(4)	0.50 ± 0.07	24.6 ± 4.46	0.05 ± 0.01 (4)	1.0 ± 0.16	22.8 ± 5.5
Gallamine	0.83 ± 0.13(4)	0.56 ± 0.07	0.7 ± 0.12	0.57 ± 0.06 (4)	1.73 ± 0.43	3.3 ± 1.11
Pancuronium	0.02 ± 0.002(4)	0.08 ± 0.01	5.2 ± 0.08	0.007 ± 0.001(4)	0.08 ± 0.02	12.6 ± 3.7
Fazadinium	0.69 ± 0.15(4)	0.29 ± 0.09	0.5 ± 0.11	0.22 ± 0.02 (4)	0.70 ± 0.01	0.9 ± 0.41

[1] Dose in mg/kg iv producing 50 % neuromuscular blockade ± SEM where shown.
[2] Dose in mg/kg iv producing 50 % vagal blockade ± SEM where shown.
[3] No. of animals.

Compound **1a** was less potent in monkeys than in cats (Tables I and II). Increase in inter-onium chain length in compounds **1i-1n** increased neuromuscular blocking activity in both species, and with compounds **1k** and **1l**, the relative increase in potency was similar in monkeys and cats (Fig. 1). Although potencies of **1m** and **1n** were not recorded in the monkey, the similarity of **1k** and **1l** in cats and monkeys suggests that maximum potency is probably reached at this point in both species. Thus, optimum activity is reached in this series with an inter-onium spacing of some 13-14 methylene or other spacially equivalent groups. This characteristic is in accord with simpler polymethylene-*bis*-ethonium and polymethylene-*poly*-ethonium compounds, which are also competitive blockers, and contrasts with the optimum inter-onium spacing of 10-11 units in the depolarising polymethylene-*bis*-methonium series (11).

The tendency to cause vagal blockade at neuromuscular blocking doses was markedly reduced in **1k** and **1l** with VD50/PD50 in monkeys greater than 30 (Table 2). For this and other reasons, **1k** was chosen for more detailed study and development (12) as a neuromuscular blocking agent offering a number of potential advantages over similar agents in present clinical use (13).

Replacement of the central methylene group of the inter-onium chain in **1k** by an ether oxygen, as in **1q**, had little effect on vagal block, but reduced the paralysing potency threefold. This potency drop accords with Taylor's results (14).

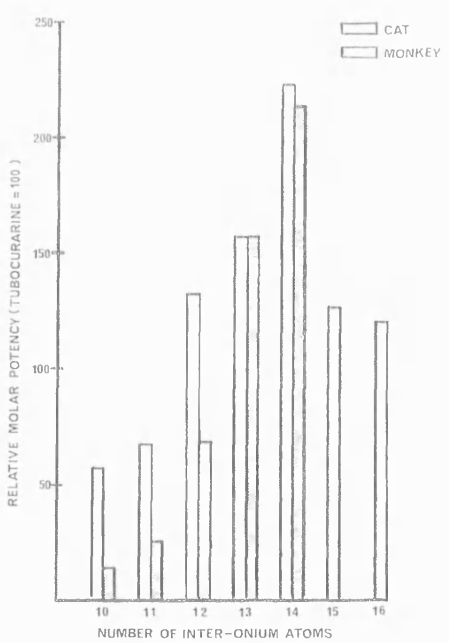


Fig. 1

In a series of depolarising neuromuscular blocking agents of related structure (15), the introduction of a benzene ring into the middle of the chain resulted in an increase in potency. In the present series, the opposite effect was observed, **1r** being less potent than **1k**. Thus structure/potency relationships in depolarising agents may not be directly applicable to non-depolarising compounds.

The compound **1k** as the besylate salt (**1k**, $X^- = Ph.SO_2O^-$), designated Atracurium Besylate (British Approved Name), has been confirmed as a potent, non-depolarising (competitive) neuromuscular blocking agent in cats and rhesus monkeys (16). Similar effects have been demonstrated in anaesthetised man (17 and 17 a-c) with no cardiovascular effects at the doses required for neuromuscular paralysis in the range 0.2 to 0.6 mg/kg.

The structure of atracurium (**1k**, m = 2) embodying, in duplicate, the two-carbon separation between quaternary nitrogen and ester carbonyl (m = 2) provides the basis for mutual promotion of Hofmann elimination and ester hydrolysis (Scheme 3). In a preliminary experiment in cats, similar substantial falls in potency were observed when atracurium was incubated for 30 min at 37°C with pH 7.4 buffer, cat and human plasma from two subjects, respectively (Table III). It is evident, therefore, that the extent and rate of breakdown of atracurium in buffer contrasts sharply with that recorded for Suxamethonium which is only 6 % hydrolysed in pH 7.4 buffer in one hour, and no more than 12 % hydrolysed in the same time at pH 7.7 (18).

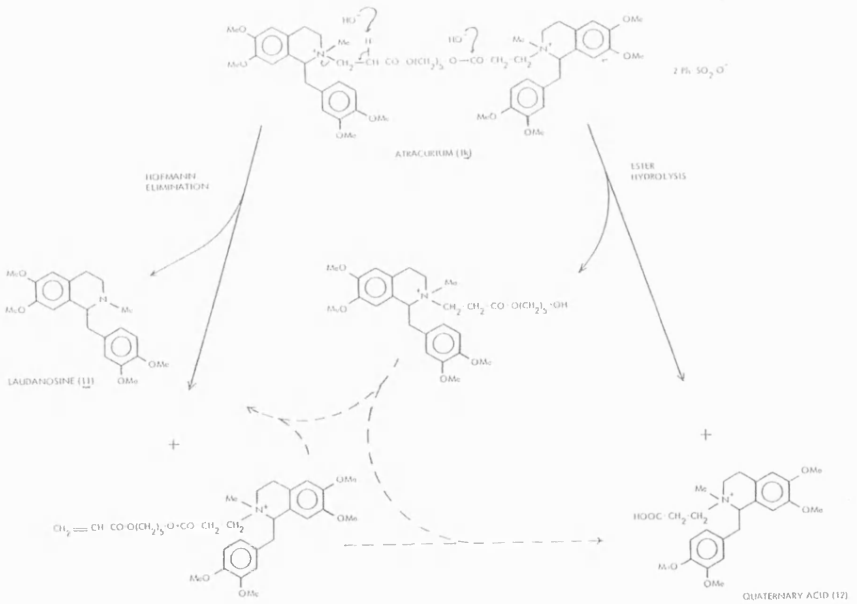
TABLE III. — *Neuromuscular blocking potency of atracurium besylate (in cats) following incubation at 37°C in pH 7.4 buffer, cat and human plasma for 30 minutes.*

Medium	PD50[1]
Cat plasma	0.32(1)[2]
Human plasma (Subject 1)	0.48(1)
Human plasma (Subject 2)	0.31(1)
Buffer pH 7.4	0.30(1)
Control[3]	0.09(4)

[1] Dose in mg/kg iv producing 50 % neuromuscular paralysis.
[2] No. of animals.
[3] Solution not incubated; acidified to pH 3.0.

Experiments with potency measurements in mice following incubation with appropriate buffers showed that atracurium undergoes non-enzymic decomposition which is three times faster at pH 7.6 than at pH 6.9 (19) indicating that chemical breakdown contributes substantially to the termination of neuromuscular blockade. Furthermore, similar *in vitro* experiments showed that the rate of decomposition in human plasma was twice as rapid as that in buffer at the same pH, indicating the involvement of an enzyme-catalysed pathway.

Evidence for the non-enzymic breakdown was further supported by the significant reduction ($P < 0.01$) of neuromuscular paralysing potency and recovery time in eight anaesthetised cats after the pH of arterial blood was increased from 7.31 to 7.63 by hyperventilation (20).



Scheme 3

Preliminary studies with ^{14}C-labelled atracurium iodide (21), prepared by methylation of the tertiary base **3** (m = 2, x = 1, A = 0) with ^{14}C-methyl iodide confirms that a base-activated Hofmann elimination at physiological pH is a major biodegradation pathway. Thus, incubation of the labelled compound in both normal human plasma and whole blood at 37°C, leads to a rapid release of some 50 % of the radiolabel as ^{14}C-laudanosine **11**. The subsequent sharp change in the slope of the curve (Fig. 2) is consistent with an initial fast Hofmann elimination of the intact ester accompanied by ester hydrolysis, with a reduction in the elimination rate as the quaternary acid **12** is formed. Similar results were obtained in atypical human plasma deficient in pseudocholinesterase (35 % of normal activity), which suggests that in contrast to Suxamethonium, the inactivation of atracurium is not dependent on this enzyme.

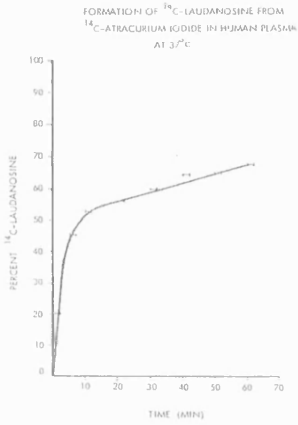


Fig. 2

Direct comparison of atracurium besylate, **1k** (m = 2, A = 3) with compound **10** (m = 3, A = 1) demonstrates the role of the interquaternary chain structure in determining potency, vagal block and metabolism (Table IV). Inter-onium group spacing is identical in both compounds (13 methylene or other spacially equivalent units), and accordingly no difference is observed in either potency (PD50) or vagal block (VD50). Compound **10** with its trimethylene separation between ester carbonyl and onium groups, is markedly less activated for both Hofmann elimination and ester hydrolysis than atracurium. In contrast to atracurium, this compound shows a significant increase in the time taken for full recovery from the onset of complete neuromuscular block ($P < 0.01$).

Studies of the effects of simulated renal failure on the action of atracurium in anaesthetised cats (in comparison with gallamine ; 20) underline the importance of its facile metabolic breakdown. These show that in the absence of renal excretion (bilateral renal ligation), the neuromuscular paralysing potency and the time-course of action of atracurium were not significantly changed, whereas the potency of gallamine was increased about twofold and recovery considerably delayed.

TABLE IV. — *Direct comparison of neuromuscular blocking potency, vagal blockade and recovery time of atracurium besylate,* **1k** (*m = 2, A = 3*) *and compound* **10** (*m = 3, A = 1*) *in groups of 6 cats.*

	Atracurium	Compound 10
PD50[1]	0.13 ± 0.01	0.16 ± 0.02[2]
VD50[1]	3.08 ± 0.76	2.78 ± 0.44[2]
$VD50/_{PD50}$	24.4 ± 6.1	18.8 ± 3.9[2]
Total recovery time[3] (duration + recovery)	22.2 ± 2.9	51.4 ± 5.2[4]

[1] Dose in mg/kg i.v. (mean ± SEM).
[2] Difference not significant.
[3] Time in minutes following standard dose of 0.25 mg/kg (mean ± SEM).
[4] $P < 0.01$.

EXPERIMENTAL

Unless stated otherwise, melting points were recorded on a Kofler Heizbank 184321 melting point apparatus. Infrared spectra were obtained on a Perkin-Elmer 157 instrument using either liquid films or in KCl discs (for solids). Proton magnetic resonance spectra were recorded on a Perkin-Elmer R12 instrument operating at 60 MHz. TMS was used as internal standard. IR and NMR data were in accord with the structures given and are available on request from the authors (RDW and GHD). Microanalytical results (C,H,N except where stated otherwise) were within ± 0.4 % of theoretical.

1,4-Bishydroxymethylbenzene

Terephthaloyl chloride (20 g ; 0.10 mole) in a mixture of dry tetrahydrofuran (80 ml) and dry ether (50 ml) was added dropwise over 0.5 h to a stirred slurry of lithium aluminium hydride (20 g ; 0.53 mole) in dry ether (250 ml). The mixture was refluxed for 4 h, cooled to 5°C, and the complex and excess hydride destroyed by the careful addition of water (20 ml), sodium hydroxide solution (5N ; 15 ml) and water (70 ml) respectively (22). The granular mass was filtered off and the filtrate evaporated *in vacuo* to leave a colourless solid which was triturated with ether (50 ml), filtered off and dried. The diol (13.5 g ; 84 %) had m.p. 115°C [Lit (23) 115-116°C and 117.5-118.5°C (24)].

3-Methylpentan-1,5-diol

3-Methylglutaric anhydride (25 g ; 0.20 mole) in a mixture of dry tetrahydrofuran (100 ml) and dry ether (100 ml) was added dropwise over 0.5 h to a stirred slurry of lithium aluminium hydride (25 g ; 0.66 mole) in dry ether (150 ml) at 5°C. The mixture was refluxed for 6 h, cooled to 0-5°C, and the complex and excess hydride destroyed by careful addition of water (25 ml), sodium hydroxide solution (5N ; 18.5 ml) and water (87.5 ml) respectively (22). The inorganic salts were filtered off, the solvents removed *in vacuo*, and the oil distilled. The diol (7.6 g ; 33 %) had b.p. 110-112.5°C/0.77 mmHg [Lit (25) b.p. 117°C/0.4 mmHg].

Alkane-αω-diol diacrylates (**5** ; Table V)

Acryloyl chloride (0.2 mole) in dry benzene (60 ml) was added over 0.5 h to a mechanically stirred mixture of the appropriate alkane-αω-diol (0.1 mole) in dry benzene (100 ml) containing tri-

ethylamine (0.2 mole) and pyrogallol (0.1 g). Further quantities of benzene (*ca* 100 ml) and triethylamine (10 ml) were added and the mixture stirred at 50°C for 0.5 h. The triethylamine hydrochloride was filtered off, and the solvent removed *in vacuo* to leave dark yellow or pale red oils which were distilled under vacuum in the presence of a trace of *p*-methoxyphenol and excluding light. After distillation, the products were stored in the dark. Data relating to the liquid acrylates prepared by this method are presented in Table V.

TABLE V

Compound No.	b.p. (°C/mmHg)	% Yield	Lit b.p. and reference	
			b.p. (°C/mmHg)	Reference
5a	113–120/11	68	102/2	10
b	132–138/13	74	73–86/0.6	26
c	90–95/0.1	61	110/1	10
d	158–165/5	33	130/1	10
e	110–113/0.03	62	"	"
f	121–122/0.02	47	"	"
g	96–99/0.1	40	118/0.8	10
h	89–93/0.4	50	"	"

1,4-Dixylidyl diacrylate

1,4-Bishydroxymethylbenzene (12 g ; 0.09 mole) and acryloyl chloride (13.8 ml ; 0.018 mole) in dry tetrahydrofuran (60 ml) and dry ether (30 ml) were treated with triethylamine (24.2 ml ; 0.18 mole) in dry ether (60 ml) over 0.5 h, with mechanical stirring. A further quantity of dry ether (100 ml) was added followed by triethylamine (10 ml), and the mixture stirred for a further 0.5 h. The triethylamine hydrochloride was filtered off and the solvents removed *in vacuo*. The yellow solid diacrylate crystallised from petroleum ether (b.p. 60-80°C) as pale yellow needles (6.3 g ; 29 %), m.p. 72-73°C [Lit (10) 74°C].

3,4-Dichlorophenylacetic acid

Prepared from 3,4-dichlorobenzyl chloride by the method of MAY and MOSETTIG (27) had m.p. 79-80°C, from petroleum ether (b.p. 60-80°C) ; [Lit (27) m.p. 82-82.5°C].

2-Bromo-4,5-dimethoxyphenylacetic acid

Bromine in chloroform (20 g in 100 ml ; 0.012 mole) was added dropwise to a solution of 3,4-dimethoxyphenylacetic acid (20 g ; 0.012 mole) in chloroform (50 ml) over 2 h with stirring. The organic layer was washed with dilute sodium thiosulphate solution (2 × 100 ml) and then with dilute sodium bicarbonate solution (250 ml, 150 ml). The combined bicarbonate washings were washed with ether (50 ml) and acidified with dilute hydrochloric acid. The colourless solid which precipitated was filtered off, washed with water, and dried *in vacuo* over P_2O_5 at 50°C. 2-Bromo-4,5-dimethoxyphenylacetic acid (22.5 g ; 80 %), m.p. 118°C, crystallised as fine needles from petroleum ether (b.p. 60-80°C)-ethyl acetate, m.p. 118-119°C [Lit (28, 29) 115-116°C and 114-116°C respectively].

N-(2-Phenylethyl)phenylacetamides (6 ; Table VI)

The amides prepared from various substituted phenylacetic acids were synthesised by the following general method. The phenylacetic acid (1 mole equivalent) and appropriate 2-phenylethylamine (1.025 mole equivalent) were heated together in an open vessel at 190-210°C for 2 h. The crystalline mass obtained on cooling the reaction mixture was purified by recrystallisation from ethanol or aqueous ethanol (except where otherwise stated in Table VI) as colourless needles. Relevant data for various amides prepared by this method are presented in Table VI.

TABLE VI

Compound No.	m.p. (°C)	% Yield	Lit m.p. and reference/Anal.	
			m.p. (°C)	Reference
6a	113	75	108 110	39 31
b	136–137	82	123.5	32
c	118–120	92	125	
d	100.5–101[1]	85	Anal. for $C_{20}H_{25}NO_5$: C,H,N	
e	141–142	73	142–144	33
f	100–101	71	101.5–102	39
g	158	80	Anal. for $C_{20}H_{24}BrNO_3$: C,H,N,Br	
h	128[2]	61[3]	Anal. for $C_{18}H_{19}Cl_2NO_3$: C,H,N	
i	135–136	69	136	15

[1] Recrystallised from ethyl acetate.
[2] Recrystallised from petroleum ether (b.p. 100-120°C).
[3] Recrystallised from petroleum ether (b.p. 80-100°C).

N-[2-(3,4-Dimethoxyphenyl)ethyl]-3(-3,4-dimethoxyphenyl)propionamide

3-(3,4-Dimethoxyphenyl)propionic acid (10 g ; 0.05 mole) and homoveratrylamine (9 g ; 0.05 mole), treated exactly as described for the preparation of the *N*-(2-phenylethyl)phenylacetamides, gave the amide (10.5 g ; 57 %, m.p. 102-104°C [Lit (36) 98-99°C].

Substituted 1-benzyl-1,2,3,4-tetrahydro-6,7-dimethoxyisoquinolines (4, Table VII)

These were prepared by a general method given in detail for the synthesis of 1,2,3,4-tetrahydro-6,7-dimethoxy-1-[2-(3,4-dimethoxyphenyl)ethyl]isoquinoline as follows : *N*-[2-(3,4-dimethoxyphenyl) ethyl]-3-(3,4-dimethoxyphenyl)propionamide (9.0 g ; 0.024 mole) in dry toluene (60 ml) was refluxed with phosphoryl chloride (40 ml) for 3 h. The partially cooled reaction mixture was then poured into petroleum ether (b.p. 60-80°C ; 400 ml), and the liquid was decanted from the semi-solid which precipitated over 15 min. The semi-solid was washed well with petroleum ether (b.p. 40-60°C), dried, and crystallised from ethanol-ether. This 3,4-dihydroisoquinolinium salt in ethanol (95 % ; 60 ml) was treated with sodium borohydride (4.0 g ; 0.105 mole) in portions over 1 h, and the mixture was then stirred for 18 h at room temperature. The excess of borohydride was then destroyed by the addition of hydrochloric acid (2N), and the mixture evaporated to dryness *in vacuo*. The off-white mass remaining was treated with sodium hydroxide solution, and the liberated amine extracted with ether (2 × 200 ml). The combined extracts were washed with water (2 × 50 ml), dried (Na_2SO_4), filtered, and treated with an ethereal hydrogen bromide solution. Crystallisation of the precipitated solid from methanol gave *1,2,3,4-tetrahydro-6,7-dimethoxy-1-[2-(3,4-dimethoxyphenyl)ethyl]isoquinoline hydrobromide* as colourless needles (4.2 g ; 40 %), m.p. 167°C. The hydrochloride, prepared from base purified *via* the hydrobromide salt, had m.p. 184°C [Lit (36) m.p. 185-186°C]. The free base was a colourless, viscous oil.

Data for the 1-benzyl-1,2,3,4-tetrahydroisoquinolines prepared by this method are given in Table VII. Where appropriate, oxalates were prepared by addition of ethereal oxalic acid solution at the relevant stage of the synthesis.

Propane-1,3-bis(4-bromobutanoate)

A γ-butyrolactone (36 g) and propane-1,3-diol (15.2 g) mixture at 0-5°C was saturated over 2 h with hydrogen bromide gas, and then left at 0°C for 24 h. The mixture was added to water (300

TABLE VII

Compound No.	Proton salt				
	Crystallising solvent	% Yield	m.p. (°C)	Lit m.p. and reference/Anal.	
				m.p. (°C)	Reference
4a[1]	MeOH-Et_2O	74	215[1]	215[1]	37
b	MeOH-Et_2O	65	177-179[1]	182[1]	32
c[3]	MeOH-Et_2O	71	218[1]	217-219[1]	35
d	EtOH	68	207-209[2]	Anal. for $C_{22}H_{27}NO_8$: C,H,N	
e	MeOH	69[3]	142-146[1]	144-146[1]	39
f[3]	MeOH	97[3]	131-132[1]	128-130[4]	35
g[5]	MeOH	56[5]	226[1]	Anal. for $C_{20}H_{25}BrClNO_4.H_2O$: C,H,N	
h	EtOH then EtOH-Et_2O	48	218-219[2]	Anal. for $C_{20}H_{24}Cl_2NO_8$: C,H,N	
i	MeOH-Et_2O	35	238[1]	236[1]	39

[1] Hydrochloride.
[2] Oxalate.
[3] Free base partially crystallised on standing.
[4] Free base, m.p. 120°C (petroleum ether, b.p. 80-100°C - ethyl acetate). [Lit (41) m.p. 111°C, aqueous ethanol].
[5] Yield after borohydride destruction, evaporation of solvents, and crystallisation of the hydrochloride from aqueous solution.

ml), and extracted with ethylene dibromide (2 × 100 ml). The combined extracts were washed with water, dried (Na_2SO_4), and evaporated to leave an oil. The major distillable component (*ca* 50 g ; b.p. 106-140°C/0.05 mmHg) was 3-bromo-1-propyl 4-bromobutanoate. The viscous pot residue was extracted with petroleum ether (60-80°C ; 3 × 150 ml) and the combined extracts evaporated to leave a colourless oil (12.5 g ; 17 %) shown by IR and NMR data to be propane-1,3-bis-(4-bromobutanoate).

NN'-5,9-Dioxa-4,10-dioxotridecylane-1,13-bis-tetrahydropapaverine, **9**

Propane-1,3-bis(4-bromobutanoate)e (1.8 g) in refluxing dry toluene (10 ml) was treated with tetrahydropapaverine (6.8 g) in toluene (50 ml) dropwise over 0.5 h. The mixture was refluxed for 18 h, cooled, and filtered from tetrahydropapaverine hydrobromide. The filtrate was evaporated *in vacuo*, and the residual oil dissolved in chloroform (10 ml). Addition of ether (*ca* 500 ml), followed by saturated ethereal oxalic acid solution (*ca* 500 ml), gave a flocculent white precipitate which was filtered off, washed with ether, and dried. Crystallisation from ethanol (twice) gave *NN'-5,9-dioxa-4,10-dioxotridecylene-1,13-bis-tetrahydropapaverine dioxalate* (2.6 g ; 49 %) as a white powder, m.p. 107-115°C.

The free *base*, **9**, obtained by ether extraction, was a colourless, viscous oil. The TLC on Polygram Sil G/UV_{254} in ethanol : ethyl acetate (1:1) gave a single spot, R_f *ca* 0.65.

1,13-Bis-{1,2,3,4-tetrahydro-6,7-dimethoxy-1-[2-(3,4-dimethoxyphenyl)ethyl]-isoquinolin-2-yl}-4,10-dioxa-3,11-dioxotridecane, **7**, *and bisquaternary salt*, **8**

1,2,3,4-Tetrahydro-6,7-dimethoxy-1-[2-(3,4-dimethoxyphenyl) ethyl]isoquinoline (3.4 g ; 0.01 mole) and pentane-1,5-diol diacrylate (1.0 g ; 0.005 mole) in dry benzene (16 ml) were stirred for 48 h under reflux and excluding light. The solvent was removed *in vacuo* and the residual oil dissolved in chloroform (15 ml). Ether (300 ml) was then added followed by a saturated solution of oxalic acid dihydrate in ether (*ca* 300 ml). The precipitated solid was filtered off, washed with ether, and dried *in vacuo* over P_2O_5. The **7** dioxalate (2.8 g ; 53 %) was obtained as a yellow powder, m.p. 69-75°C, from ethanol.

The free *base*, **7**, obtained by ether extraction, was a colourless, viscous oil. Tlc on polygram Sil G/UV_{254} in ethanol-ethyl acetate (1:1) showed a single spot, R_f *ca* 0.6.

The *NN'-dimethyl dimesylate*, **8**, was obtained by treating scrupulously dry bis-amine, **7** (0.3 g ; 0.0003 mole) in spectroscopically pure acetonitrile (1.5 ml) with freshly-distilled methyl methanesulphonate (1.0 ml) at room temperature for 48 h, maintaining anhydrous conditions throughout. The filtered reaction mixture was added dropwise to mechanically stirred, filtered, dry ether (*ca* 500 ml). The flocculent white precipitate was washed with dry ether, and dried *in vacuo* over P_2O_5 at 50°C. The bis-quaternary *salt*, **8** (0.27 g ; 73 %) had m.p. 98-105°C. *Anal.* for $C_{39}H_{52}N_2O_{10}S_2 \cdot 3H_2O$: C, H, N.

Bis-1-benzyltetrahydroisoquinoline esters, **3**

The bis-amine esters, **3**, were synthesised from the appropriate 1-benzyl-1,2,3,4-tetrahydroisoquinoline, **4**, and diol diacrylate, **5**, by the method outlined above for the preparation of 1,13-bis-{1,2,3,4-tetrahydro-6,7-dimethoxy-1-[2-(3,4-dimethoxyphenyl)ethyl] isoquinolin-2-yl}-4,10-dioxa-3,11-dioxotridecane, **7**. The *dioxalate salts* were crystallised from ethanol and recrystallised, where necessary, from the same solvent until the free *base* showed a single spot by TLC using polygram Sil G/UV_{254}, ethanol : ethyl acetate (1:1), with iodoplatinate visualisation (42). The dioxalate salts were characterised by their melting points and IR characteristics (Table VIII). The free base, obtained from pure dioxalate salt, was isolated with ether or preferably toluene.

TABLE VIII

Compound No.	Base m.p. (°C)	Dioxalate salt	
		% Yield from diacrylate	m.p. (°C)
3a	47-49	69	124-128[1]
b	Oil	64	130-136
c	Oil	70	124-126
d	44-46	34	113-117
e	44-46	38	129-137
f	46-47	42	124-128
g	65-67	48	128-133
h	45-48	25	130-138
i	46-48	50	126-132
j	44-46	48	123-126
k	Oil	51	117-121[2]
l	Oil	67	115-119
m	Glass	76	112-116
n	Glass	60	124-135
o	49-50	68	146-151
p		39	120-127
q		57	113-118
r		35	128-135
s		38	112-118

[1] *Anal.* for $C_{58}H_{70}N_2O_{20}$, C, H, N.
[2] *Anal.* for $C_{52}H_{64}N_2O_{28}$, H_2O, C, H, N.

The *NN'-dimethyl quaternary salts* (**1a-1s** and **10**, Table IX) were prepared and isolated in a similar way to that described for synthesis of *NN'*-dimethyl-1,13-bis-{1,2,3,4-tetrahydro-6,7-dimethoxy - 1 - [2-(3,4-dimethoxyphenyl)ethyl]isoquinolinium-2-yl}-4,10-dioxa-3,11-dioxotridecane dimesylate (**8**, above) using scrupulously dry bis-amine and purified methyl iodide or methyl methanesulphonate, as appropriate. ^{14}C-Atracurium iodide was prepared similarly from the base **3k** and ^{14}C-methyl iodide.

TABLE IX

Compound No.	Salt	% Yield from Bis-amine,)	m.p. (°C)	Formula	Microanalysis
1a	Mesylate	90	100–110	$C_{52}H_{72}N_2O_{18}S_2.2H_2O$	C,H,N
b	Iodide	95	141–145	$C_{46}H_{58}I_2N_2O_8.1H_2O$	C,H,N
c	Iodide	90	143–150	$C_{48}H_{62}I_2N_2O_{10}.1H_2O$	C,H,N
d	Mesylate	91	Softens 86 Softens	$C_{52}H_{72}N_2O_{18}S_2.2.5H_2O$	C,H,N
e	Mesylate	87	121	$C_{50}H_{64}N_2O_{18}S_2.1.5H_2O$	C,H,N
f	Mesylate	81	Softens 123	$C_{54}H_{76}N_2O_{20}S_2.1.5H_2O$	C,H,N
g	Mesylate	71	Softens 128 Softens	$C_{52}H_{70}Br_2N_2O_{18}S_2.2H_2O$	C,H,N
h	Mesylate	70	111	$C_{48}H_{60}Cl_4N_2O_{15}S_2.0.5H_2O$	C,H,N
i	Mesylate	85	96–120	$C_{53}H_{74}N_2O_{18}S_2.1H_2O$	C,H,N
j	Mesylate	89	91–115	$C_{54}H_{76}N_2O_{18}S_2.4H_2O$	C,H,N
k	Iodide	86	143–148	$C_{51}H_{72}I_2N_2O_{12}$	C,H,N
	Mesylate	80	105–112	$C_{55}H_{78}N_2O_{18}S_2.2H_2O$	C,H,N
	Besylate	85	85–90	$C_{63}H_{82}N_2O_{18}S_2.4H_2O$	C,H,N,S
	Tosylate	85	70–90	$C_{67}H_{86}N_2O_{18}S_2.2H_2O$	C,H,N,S
	1-Napsylate	85	65–85	$C_{71}H_{86}N_2O_{18}S_2.3H_2O$	C,H,N,S
	2-Napsylate	85	60–80	$C_{71}H_{86}N_2O_{18}S_2.2H_2O$	C,H,N,S
l	Iodide	88	132–138	$C_{54}H_{74}I_2N_2O_{12}.2H_2O$	C,H,N
	Mesylate	84	109–116	$C_{56}H_{80}N_2O_{18}S_2.1H_2O$	C,H,N
m	Iodide	92	119–123	$C_{55}H_{76}I_2N_2O_{12}$	C,H,N
n	Iodide	82	119–123	$C_{56}H_{78}I_2N_2O_{12}$	C,H,N
o	Iodide	85	114–148	$C_{48}H_{58}I_2N_2O_{12}$	C,H,N
p	Iodide	80	122–129	$C_{51}H_{64}I_2N_2O_{12}$	C,H,N
q	Iodide	81	119–128	$C_{52}H_{70}I_2N_2O_{13}$	C,H,N
r	Mesylate	83	117–127	$C_{58}H_{76}N_2O_{18}S_2$	C,H,N
s	Mesylate	79	100.5–109	$C_{56}H_{80}N_2O_{18}S_2.1H_2O$	C,H,N
10	Mesylate	84	95–102	$C_{52}H_{78}N_2O_{18}S_2.3H_2O$	C,H,N

Studies with ^{14}C-Atracurium iodide in normal human plasma in vitro

^{14}C-Atracurium iodide in methanol (1 ml ; 100 μg/ml) was placed in a test tube, and the methanol removed in a current of nitrogen at 37°C. Freshly reconstituted pooled Dried Human Plasma (5 ml ; pH 7.4) was added, mixed, and the solution maintained at 37°C with gentle shaking. Plasma samples (0.6 ml) were taken at timed intervals, extracted with ether (5 ml), and appropriate aliquots taken for counting. Controls were effected by repeating the experiment in duplicate using water (5 ml) in place of plasma, and extracting immediately with ether (5 ml). The results in a typical experiment are shown in Fig. 2. Similar results were obtained with a sample of fresh whole blood and fresh plasma from a normal subject ; also with atypical human plasma with a 65 % deficiency in pseudocholinesterase.

Neuromuscular and vagal blocking potencies

Neuromuscular and vagal blocking potencies were determined on gastrocnemius muscle-sciatic nerve preparations from cats and rhesus monkeys as previously described (43, 44). The results are given in Tables I and II.

Stability of Atracurium besylate in pH 7.4 buffer, cat and human plasma

Atracurium besylate was incubated with 0.2M tris buffer pH 7.4, cat and human plasma (2 subjects) respectively at 37°C for 30 min. The solution was adjusted to pH 4.2 with glacial acetic acid, cooled in ice, and its neuromuscular blocking potency determined in the cat as described above (1). The results are given in Table III.

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RÉSUMÉ

Une série de di-esters d'α,ω-bisbenzylisoquinolinium destinés à subir une élimination d'Hofmann au pH physiologique a été synthétisée. La puissance du blocage neuromusculaire augmente jusqu'à un espacement de 13 ou 14 atomes, pour diminuer ensuite. Il a été démontré que les composés les plus actifs possèdent une bonne activité de blocage neuromusculaire non-dépolarisante avec une forte spécificité envers la jonction neuromusculaire et une durée d'action moyenne. Le Bésylate d'Atracurium (dibenzène-sulfonate di-isoquinolinium [1,1', 2,2', 3,3', 4,4'-octahydro-6,6', 7,7'-tétraméthoxy-2,2'-diméthyl-1,1'-divératryl-2,2'-(3,11-dioxo-4,10-dioxatridéca-méthylène)] qui est en cours d'étude clinique comme agent de blocage neuromusculaire semble être rendu inactif par des mécanismes enzymatiques et non-enzymatiques. Il semble que la voie chimique de l'élimination d'Hofmann joue un rôle important dans cette inactivation.

ZUSAMMENFASSUNG

Eine Reihe α,ω-Bisbenzylisochinolinium-polyalkylen-diester, die bei physiologischem pH einer Hofmann-Eliminierung unterliegen sollten, wurde synthetisiert. Die neuromuskulär blockierende Wirkung steigt bis zu einer Interonium-Distanz von 13 bis 14 Atomen, darüber nimmt sie wieder ab. Die wirksamsten Verbindungen zeigten gute nicht depolarisierende neuromuskuläre Aktivität mit hoher Spezifizität für die neuromuskuläre Junktion und mittlerer Wirkungsdauer. Atracurium Besylat (1,1',2,2',3,3',4,4'-Octahydro-6,6',7,7',-tetramethoxy-2,2'-dimethyl-1,1'-diveratryl-2,2'-(3,11-dioxo-4,10-dioxatridecamethylen)-diisochinolinium-dibenzosulfonat) wurde für klinische Zwecke entwickelt zur neuromuskulären Blockierung. Es wird offenbar sowohl durch enzymatische als auch nicht-enzymatische Mechanismen inaktiviert. Es gibt Anzeichen für die Vermutung, daß die chemisch erwartete Hofmann-Eliminierung eine wichtige Rolle in dieser Inaktivierung spielt.

REFERENCES

(1) J.B. STENLAKE, J. URWIN, R.D. WAIGH and R. HUGHES, *Eur. J. Med. Chem.*, 1979, **14**, 77.
(2) J.B. STENLAKE, J. URWIN, R.D. WAIGH and R. HUGHES, *Eur. J. Med. Chem.*, 1979, **14**, 85.
(3) J.B. STENLAKE, R.D. WAIGH, J. URWIN, G.H. DEWAR, R. HUGHES and D.J. CHAPPLE, *Eur. J. Med. Chem.*, 1981, **16**, 000.
(4) H.O.J. COLLIER, J.M.Z. GLADYCH, B. MACAULAY and E.P. TAYLOR, *Nature (London)*, 1958, **182**, 1424.
(5) E.D. BERGMANN, D. GINSBURY and R. PAPPO, *Organic Reactions*, Eds R. Adams *et al.*, Wiley, New York, 1959, **10**, 179.
(6) A. BISCHLER and B. NAPIERALSKI, *Ber.*, 1893, **26**, 1903.
(7) W.M. WHALEY and T.R. GOVINDACHARI, *Organic Reactions*, Eds. R. Adams *et al.*, Wiley, New York, 1951, **6**, 74.
(8) J.S. BUCK, R.D. HAWORTH and W.H. PERKIN, *J. Chem. Soc.*, 1924, 2176.
(9) L.E. CRAIG and D.S. TARBELL, *J. Am. Chem. Soc.*, 1948, **70**, 2783.
(10) F. HENRY and H. JULLIEN, *C. R. Acad. Sci., Paris*, 1972, **274**, 639.
(11) R.B. BARLOW and A. ZOLLER, *Br. J. Pharmacol.*, 1964, **23**, 131.
(12) J.B. STENLAKE, R.D. WAIGH, G.H. DEWAR, J. URWIN and N.C. DHAR. U.K. Patent, 1980, N° 1.579.822 ; Belgian Patent, 1977, N° 76355 ; U.S. Patent, 1979, N° 4,179,507.

(13) J.B. Stenlake, *Adv. in Pharmacol. and Ther.*, Volume 3. Ions. Cyclic Nucleotides. Cholinergy. Ed. J.C. Stoclet, Pergamon Press, Oxford and New York, 1979, 303.
(14) E.P. Taylor, *J. Chem. Soc.*, 1952, **142**, 1309.
(15) A.F. Danilov, I.J. Kvitko, V.V. Lavrentieva, M.J. Michelson, B.A. Porai-Koshits, E.K. Rozhkova and S.A. Shelkovnikov, *Br. J. Pharmacol.*, 1972, **44**, 765.
(16) R. Hughes and D.J. Chapple, *Br. J. Anaesth.*, 1980, **52**, 238P.
(17) T.M. Hunt, R. Hughes and J.P. Payne, *Br. J. Anaesth.*, 1980, **52**, 238P.
(17a) R. Hughes, T.M. Hunt and J.P. Payne, *Br. J. Anaesth.*, 1980, **52**, 634P.
(17b) G.G. Coker, G.H. Dewar, R. Hughes, T.M. Hunt, J.P. Payne, J.B. Stenlake and R.D. Waigh, *Acta anaesth. scand.*, 1981, **25**, 67.
(17c) J.P. Payne and R. Hughes, *Br. J. Anaesth.*, 1981, **53**, 45.
(18) H.W. Goedde, K.R. Held and K. Altland, *Mol. Pharmacol.*, 1968, **4**, 274.
(19) C.W. Thompson (1979), personal communication.
(20) R. Hughes and D.J. Chapple, *Br. J. Anaesth.*, 1981, **53**, 31.
(21) J.B. Stenlake, D. Watt and N.C. Dhar, unpublished.
(22) L.H. Amundsen and L.S. Nelson, *J. Am. Chem. Soc.*, 1951, **73**, 242.
(23) E. Bourquelot and A. Ludwig, *C. R. Acad. Sci., Paris*, 1914, **159**, 213.
(24) M.J. Rhoad and P.J. Flory, *J. Am. Chem. Soc.*, 1950, **72**, 2216.
(25) L. Wartski, *Bull. Soc. Chim. Fr.*, 1965, 3066.
(26) W. Reppe, A. Magin, C. Schuster, R. Keller, H. Kröper, T. Klein, F.W. Kerchow, G.V. Blank, K. Merkel, H. Scheller, L. Weschky, K. Wolff, W. Schweckendiek, O. Hecht, E. Gassenmeier and A. Simon, *Ann.*, 1953, **582**, 1.
(27) E.L. May and E. Mosettig, *J. Org. Chem.*, 1946, **11**, 631.
(28) R. Pschorr, *Ann.*, 1912, **391**, 23.
(29) L. Small and S.G. Turnbull, *J. Am. Chem. Soc.*, 1937, **59**, 1541.
(30) T.R. Govindachari and K. Nagarajan, *Proc. Ind. Acad. Sci.*, 1955, **42A**, 136 ; *Chem. Abstr.*, 1956, **50**, 7804g.
(31) R.A. Robinson, U.S. Patent 2,683,146 ; *Chem. Abstr.*, 1955, **49**, 9045a.
(32) H. Kondo and T. Kondo, *J. Pharm. Soc. Jap.*, 1928, **48**, 324 ; *Chem. Abstr.*, 1928, **22**, 3414.
(33) J.S. Buck and W.H. Perkin, *J. Chem. Soc.*, 1924, 1675.
(34) M. Tomita, M. Kozuka and S. Uyeo, *Yakugaku Zasshi*, 1966, **86**, 460 ; *Chem. Abstr.*, 1966, **65**, 10634c.
(35) A. Pictet and A. Gams, *C. R. Acad. Sci., Paris*, 1911, **153**, 386.
(36) M. Shamma and M.J. Hillman, *Tetrahedron*, 1971, **27**, 1363.
(37) R.A. Robinson, *J. Org. Chem.*, 1951, **16**, 1911.
(38) F.L. Pyman, *J. Chem. Soc.*, 1909, 1610.
(39) M. Tomita and J. Niimi, *Yakugaku Zasshi*, 1959, **79**, 1023 ; *Chem. Abstr.*, 1960, **54**, 5724g.
(40) R.D. Haworth, W.H. Perkin and J. Rankin, *J. Chem. Soc.*, 1924, 1686.
(41) T. Kametani and M. Ihara, *J. Chem. Soc., C*, 1967, 530.
(42) L.R. Goldbaum and L. Lazyak, *Anal. Chem.*, 1956, **28**, 1289.
(43) R. Hughes and D.J. Chapple, *Br. J. Anaesth.*, 1976, **48**, 59.
(44) R. Hughes and D.J. Chapple, *Br. J. Anaesth.*, 1976, **48**, 847.

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A Preliminary Assessment of Atracurium, a New Competitive Neuromuscular Blocking Agent

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Atracurium besylate, 2,2′-(3,11-dioxo-4,10-dioxatridecylene)-bis-[6,7-dimethoxy-1-(3,4-dimethoxybenzyl)-2-methyl-1,2,3,4-tetrahydroisoquinolinium] dibenzenesulphonate is a potent non-depolarising (competitive) neuromuscular blocking agent in the cat, monkey, dog and anaesthetised man. In man, it caused complete paralysis of the tetanic response of the adductor pollicis muscles at doses of 0.2 mg/kg. Blockade was of medium duration with rapid spontaneous recovery, and was readily reversed by neostigmine. The electrocardiogram, heart rate, arterial blood pressure and central venous pressure were virtually unchanged following doses of 0.2–0.4 mg/kg. Intubation was readily accomplished in 1.5–2 min after administration of 0.25–0.3 mg/kg.

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All non-depolarising (competitive) neuromuscular blocking agents in present clinical use have disadvantages. In particular, all exhibit vagal blockade and other cardiovascular effects in some degree (Hughes & Chapple 1976a), and all show a significant increase in the duration of neuromuscular blockade when excretion is inhibited by renal insufficiency (Wingard & Cook 1977).

Atracurium besylate (British approved name), 2,2′-(3,11-dioxo-4,10-dioxatridecylene)-bis-[6,7-dimethoxy-1-(3,4-dimethoxybenzyl)-2-methyl-1,2,3,4-tetrahydroisoquinolinium] dibenzenesulphonate (Fig. 1), is substantially free of these disadvantages. It is one of a series of non-depolarising neuromuscular blocking agents designed to induce ready fragmentation to inactive moieties *in vivo* by a combination of enzymic ester hydrolysis and facile base-catalysed degradation of its quaternary ammonium groups initiated at physiological pH (Stenlake et al. 1977). A preliminary account of its properties has already been reported to the Anaesthetics Research Society (Hughes & Chapple 1980, Hunt et al. 1980).

MATERIAL AND METHODS

Effects of atracurium besylate (0.5–1.0 μg/ml) were studied on isolated chick biventer-cervicis preparations, equilibrated with 95% O_2 and 5% CO_2 in Krebs solution at 35° and stimulated at 0.12 Hz with rectangular pulses of supramaximal voltage and duration. Effects on neuromuscular transmission following bolus intravenous injection of atracurium were studied on sciatic nerve-gastrocnemius muscle preparations in anaesthetised cats as described by Hughes & Chapple (1976a), by similar techniques in beagle dogs, and in rhesus monkeys (Hughes & Chapple 1976b) using the anaesthetic regime described for man. Arterial blood pressure, respiration and heart rate were recorded. Effects on autonomic nervous transmission were assessed by stimulating the cervical vagus nerve and measuring the ensuing bradycardia, and by stimulating the cervical sympathetic nerve and recording the contractions of the nictitating membrane. Histamine release was assessed in beagle dogs using H_1- and H_2-receptor antagonists.

Studies in anaesthetised man using the techniques described by Hughes et al. (1976) were performed in 12 male patients who had given their informed consent and were about to undergo urological surgery. The trial was conducted with the authority of a Clinical Trial Certificate issued with the approval of the Committee on Safety of Medicines. Approval was also obtained from the Ethics Committee of St Peter's Hospitals. No premedication was given. Anaesthesia was induced with 5% thiopentone (300–600 mg) and in 9 of the 12 patients continued with 2 to 4% halothane in oxygen until deep enough for intubation, which was carried out without the use of a neuromuscular blocking agent after the larynx had been sprayed with 4% lignocaine.

Halothane was then withdrawn and anaesthesia maintained with 60–75% nitrous oxide and oxygen combined with intermittent positive pressure ventilation. Supplements of 100–200 mg thiopentone and 25–50 mg pethidine were given as required. Tetanic and single twitch contractions of the adductor pollicis muscles were recorded simultaneously by stimulating each ulnar nerve supramaximally at the wrist every 12 s, one with tetanic bursts (50 Hz, 1 s) and the other with single shocks.

Arterial pressure and central venous pressure were measured directly from catheters in the radial artery and the right heart, respectively; heart rate was obtained from the electrocardiogram. Arterial blood gases were maintained within normal limits.

Thereafter, atracurium was given intravenously in the dose range 0.2–0.3 mg/kg. Three patients, who were not given halothane but maintained on nitrous oxide and oxygen alone, were intubated after the administration of the drug.

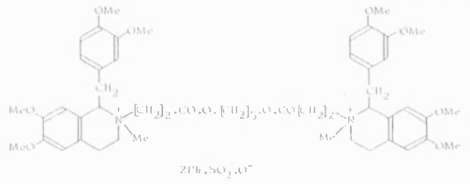

Fig. 1. Chemical structure of atracurium.

RESULTS

Atracurium caused a non-depolarising block of the chick biventer-cervicis preparation without producing initial contracture. In anaesthetised cats, intravenous doses of 0.25 mg/kg (four cats) or 0.5 mg/kg (two cats) caused full neuromuscular blockade and arrested breathing in 1–2 min; full recovery required 35±4.7 min. Edrophonium (0.2 mg/kg i.v.) and neostigmine (0.05 mg/kg i.v.) were effective antagonists. Vagal blockade only became appreciable at doses 8 times the full neuromuscular paralysing dose (Fig. 2). At these doses, sympathetic effects were slight. Arterial blood pressure was reduced by a mean of 34±5.4% only with 16 times the full paralysing dose; changes in heart rate were minimal. In six other cats, neuromuscular paralysing potency and the course of recovery were similar whether atracurium was administered via the hepatic portal vein or via the jugular vein. The action of atracurium and its duration were unaltered when renal function was abolished by ligating the artery, vein and ureter of each kidney.

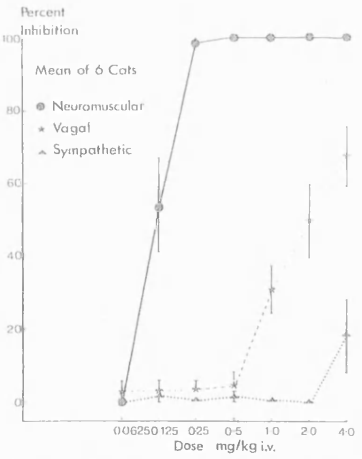

Fig. 2. Dose-response curves for atracurium showing blockade of neuromuscular and autonomic mechanisms in anaesthetised cats. Vertical lines indicate s.e.mean.

Potency and the time course of action of atracurium were similar in seven rhesus monkeys and four beagle dogs to results in cats. Appreciable vagal blockade occurred only at 16 times full neuromuscular paralysing doses in the monkey. At this dose, sympathetic effects were slight and changes in heart rate minimal, though 19±7% reduction in arterial blood pressure occurred. In four beagle dogs, only doses of 4 and 8 times the full neuromuscular paralysing dose (0.25 mg/kg i.v.) caused mean reductions respectively in arterial blood pressure of 28±14.1% and 47±20.2%, and in heart rate of 10±4.0% and 12±1.5%. Mepyramine 10 mg/kg i.v. (H_1-receptor antagonist) and burimamide 15 mg/kg i.v. (H_2-receptor antagonist) reduced the fall in arterial blood pressure, caused by 2 mg/kg atracurium (8×full paralysing dose) from 52 to 16% and 42 to 25%, respectively, in each of two dogs.

In anaesthetised man, atracurium, 0.2 mg/kg i.v. caused complete paralysis of the tetanic response of the adductor pollicis muscles; 0.3 mg/kg was required to block the single twitch response (Hunt et al. 1980). Blockade was of medium duration (6–19 min) with spontaneous recovery of the tetanic response in approx. 30 min; paralysis was readily reversed by neostigmine. No important changes in the electrocardiogram, heart rate, arterial blood pressure or central venous pressure were observed following doses of 0.2 to 0.4 mg/kg atracurium (Fig. 3). In each of three patients, intubation was accomplished in 1.5–2 min after administration of 0.25–0.3 mg/kg atracurium and when the vocal cords were relaxed. A paralysing dose of 0.2 mg/kg in one patient maintained on 1% halothane throughout did not

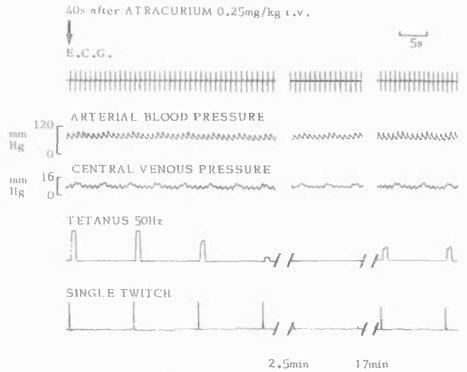

Fig. 3. Tracings from an anaesthetised patient recorded at a faster paper speed showed that an intravenous dose of 0.25 mg/kg atracurium, which abolished the tetanic and single twitch responses of the adductor pollicis muscle, did not change the electrocardiogram, heart rate, arterial blood pressure or central venous pressure.

potentiate the hypotensive effects of halothane. All 12 patients studied made uneventful recoveries.

DISCUSSION

The results in laboratory animals show that atracurium is a potent, non-depolarising neuromuscular blocking agent. It is readily antagonised by neostigmine and shows a wide separation between its neuromuscular paralysing actions and cardiovascular side-effects.

In man, the potency and duration of action of atracurium proved to be similar to those observed in laboratory studies. The tetanic response was depressed more readily than that of the single twitch, and tetanic fade was prominent. Intubation was possible within 90–120 s of injection. Recovery which followed a sigmoid pattern in both responses resembled that following suxamethonium (Sugai et al. 1975) rather than non-depolarising drugs. Neuromuscular paralysis was rapidly reversed by neostigmine. Cardiovascular stability following administration of atracurium to man was good and comparable with that of dimethyltubocurarine (Hughes et al. 1976). No important changes were observed in the electrocardiogram, heart rate, arterial blood pressure or central venous pressure (Fig. 3). The very consistent rate of recovery observed in patients is seen as a distinct advantage, which animal studies suggest may well be independent of hepatic and renal function.

REFERENCES

Hughes, R. & Chapple, D. J. (1976a) Effects of non-depolarizing neuromuscular blocking agents on peripheral autonomic mechanisms in cats. *Brit. J. Anaesth.* **48**, 59.

Hughes, R. & Chapple, D. J. (1976b) Cardiovascular and neuromuscular effects of dimethyltubocurarine in anaesthetized cats and rhesus monkeys. *Brit. J. Anaesth.* **48**, 847.

Hughes, R. & Chapple, D. J. (1980) Experimental studies with atracurium a new neuromuscular blocking agent. *Brit. J. Anaesth.* **52**, 238 P.

Hughes, R., Ingram, G. S. & Payne, J. P. (1976) Studies on dimethyltubocurarine in anaesthetized man. *Brit. J. Anaesth.* **48**, 969.

Hunt, T. M., Hughes, R. & Payne, J. P. (1980) Preliminary studies with atracurium in anaesthetized man. *Brit. J. Anaesth.* **52**, 238 P.

Stenlake, J. B., Waigh, R. D., Dewar, G., Urwin, J. & Dhar, N. C. (1977) U.K. provisional patent applications Nos 50589/75 and 45028/76; Belgian patent No. 76355.

Sugai, N., Hughes, R. & Payne, J. P. (1975) The effect of suxamethonium alone and its interaction with gallamine on the indirectly elicited tetanic single twitch contractions of skeletal muscle in man during anaesthesia. *Brit. J. clin. Pharm.* **2**, 391.

Wingard, L. B. & Cook, D. R. (1977) Clinical pharmacokinetics of muscle relaxants. *Clin. Pharm.* **2**, 330.

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Biodegradable neuromuscular blocking agents

Part. 5 - α, ω-bisquaternary polyalkylene phenolic esters

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Summary. — A small group of α,ω-bisquaternary polyalkylene esters of quinol, catechol and pyrogallol have been synthesised, and tested as potential biodegradable neuromuscular blocking agents. Three of the compounds were virtually inactive and the remainder of low potency compared to that of tubocurarine. Duration of action and vagal blockade have also been recorded.

Résumé. — Un groupe restreint d'esters polyalkeniques α, ω bisquaternaires de quinol, catéchol et pyrogallol ont été synthétisés et essayés en tant qu'agents bloquants dans le domaine neuromusculaire, biodégradables. Trois des composés sont inactifs et le dernier n'a qu'une faible activité en comparaison de celle de la tubocurarine. La durée d'action et le blocage vagal ont aussi été enregistrés.

Zusammenfassung. — Einige α,ω-bisquartäre Polyalkylen-ester von Hydrochinon, Brenzcatechin und Pyrogallol wurden synthetisiert und als potentielle abbaubare, neuromuskuläre Hemmstoffe getestet. Wirkungsdauer und Vagus-Hemmung wurden bestimmt.

Key-words : Neuromuscular blocking agents. — Quinol, catechol, pyrogallol (α, ω-bisquaternary polyalkylene esters) Quaternary ammonium derivatives. — Esters (quinol, catechol, pyrogallol). — Vagal blockade.

In previous papers (1-4), we have described a new approach to shorter acting neuromuscular blocking agents based on Hofmann elimination of appropriate quaternary ammonium salts, rather than enzymatic biotransformation. These studies led (4) to a particularly promising agent, atracurium besylate, of high neuromuscular blocking potency and exceptional freedom from side effects, but neither atracurium nor any similar compounds possessed the extreme brevity of action of succinylcholine. This paper describes attempts to shorten the duration of drug action by studying analogous quaternised 3-aminopropanoyl derivatives of quinol, catechol and pyrogallol. It was anticipated that such compounds would undergo more rapid Hofmann elimination at physiological pH than those esters reported hitherto (1-4) and also more rapid hydrolysis, both of which would contribute to shorter action and more rapid recovery.

CHEMISTRY

The quaternary salts (**3a-g**) were prepared by the route shown in Scheme 1. Quinol diacrylate **1a**, catechol diacrylate **1b** and pyrogallol triacrylate **1f** were synthesised by methods previously described (3, 4). Attempts to prepare resorcinol diacrylate and phloroglucinol triacrylate gave only polymeric material. The quinol, catechol and pyrogallol acrylates proved to be remarkably reactive acceptors in the Michael reaction with secondary amines, as with diethylamine and 1,2,3,4-tetrahydro-6,7-dimethoxyquinoline, which react rapidly at room temperature. Tetrahydropapaverine, likewise, adds readily to quinol diacrylate to yield the required di-tertiary base **2g**. However, in the reaction between tetrahydropapaverine and catechol diacrylate, IR evidence suggested extensive amidation and pure bisamine, **2**, could not be obtained.

The reaction between ethanolic dimethylamine and quinol diacrylate also resulted in some amide formation, but pure **2a** was obtained by crystallisation. Catechol diacrylate and ethanolic dimethylamine, however, failed to yield the di-tertiary base, **2h**, giving a mixture of its decomposition products, ethyl 3-dimethylaminopropionate, **4**, *N*,*N*-dimethyl-3-dimethylaminopropionamide, **5**, and colourless needles (m.p. 64 from light petroleum) of a product with IR and NMR spectroscopic properties consistent with the structure of the dimethylammonium salt of 2-(3-dimethylaminopropionoxy)phenol, **6**. NMR evidence from this reaction and the following study of the pyrogallol triacrylate reaction with diethylamine show that an initial rapid Michael addition is followed by alcoholysis and amidation as shown in Scheme 2.

The reaction between pyrogallol triacrylate and dimethylamine was followed by NMR spectroscopy. The course of the reaction was effectively followed by observing the disappearance of the olefinic protons in the δ5.80-6.80 region and the emergence of the $COCH_2CH_2N$ proton resonance at *ca* δ2.70, indicative of Michael addition.

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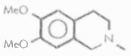
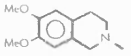
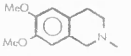
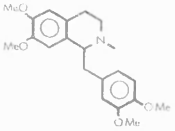
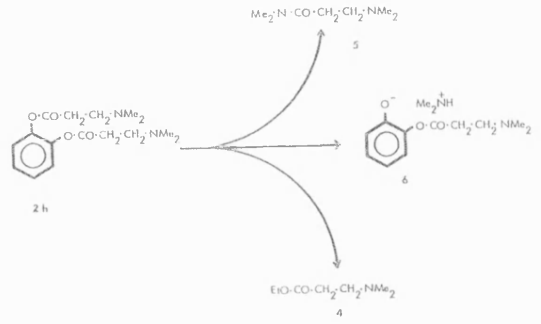
Compound	n	Substitution pattern	B	R (Compounds 3)	X^- (Compounds 3)
(a)	1	1,4	Me_2N-	Me	I^-
(b)	1	1,2	Et_2N-	H	Cl^-
(c)	1	1,4	Et_2N-	H	Cl^-
(d)	1	1,2		Me	I^-
(e)	1	1,4		Me	I^-
(f)	2	1,2,3		Me	I^-
(g)	1	1,4		Me	FSO_3^-

Scheme 1

Amide formation was observed by the appearance of the $CON(CH_3)_2$ singlets of *N,N*-dimethyl-3-dimethylaminopropionamide at δ2.97 and δ3.06, together with the associated singlet at *ca* δ8.10 for the phenolic hydroxyl group. Careful addition of dimethylamine gas to the NMR sample of pyrogallol triacrylate (0.1g) in $CDCl_3$ at 5° C gave complete Michael addition in 2.75 min. without amide formation. The 18 *N*-methyl protons appeared as a singlet at δ2.28, the 12 protons arising from the $COCH_2CH_2N$ moieties as a singlet at δ2.71 indicating equivalence of the methylene protons in the resulting tris-amine, **7**. The aromatic protons appeared as multiplet at δ7.14-7.26. However, all attempts to isolate **7** resulted in extensive alcoholysis and amidation.



Scheme 2

The driving force for the breakdown of **2h** and **7** seems to be relief of steric strain in these bulky 1,2-di- and 1,2,3-triesters. This is absent in the corresponding 1,4-quinol di-esters accounting for their stability. It is also inhibited in the 1,2- and 1,2,3-compounds derived from bulkier secondary amines, presumably due partly to shielding of the ester carbonyl by the larger 3-amino-substituents in the Michael addition products and partly to steric hindrance to attack at the ester carbonyl by the larger secondary bases.

The simple dimethylamino base, **2a**, was readily quaternised with methyl iodide in ethanol to yield **3a**. Similar treatment of the diethylamine bases, **2b** and **2c**, under a variety of conditions failed to yield the required quaternary salts. Evidence of elimination was observed not only in these unsuccessful quaternisation reactions, but also during the crystallisation of the corresponding dihydrochlorides from ethanol-ether. The ease of decomposition of these phenolic amine salts is reminiscent of the rapid decomposition observed in β-aminoketone proton salts and methiodides which are profitably employed in the Robinson modification (5, 6) of the Mannich and Michael reactions, for the *in situ* formation of acrylates.

In contrast, the 1,2,3,4-tetrahydro-6,7-dimethoxyisoquinoline derivatives, **2d**, **2e** and **2f**, were all successfully quaternised at room temperature by methyl iodide. The tetrahydropapaverine analogue, **2g**, however, could not be quaternised with methyl iodide under a variety of reaction conditions without accompanying side reactions. More reactive reagents such as dimethyl sulphate and methyl methanesulphonate, likewise, failed to yield pure material. The more powerful methyl fluorosulphonate (7), however, gave the required bis-quaternary **3g**, on fractionation of the product.

PHARMACOLOGICAL RESULTS AND DISCUSSION

Neuromuscular blocking potencies and the extent of vagal blockade of the quaternary salts, **3a** and **d-g**, were measured in anaesthetised cats (Table I). Compounds,

TABLE I. — *Neuromuscular blocking potencies and vagolytic effects of a ω-bis-quaternary polyalkylene phenolic esters in cats.*

Compound No.	Relative molar neuromuscular blocking potency[1]	PD50[2]	VD50[3]	VD50/PD50	Recovery[4] time (min)
3a	19.2	0.52	12.7	24.3	15
	16.1	0.62	4.1	6.7	20
d	0[5]	-	3.8	-	-
	0[5]	-	7.0	-	-
e	4.2	3.60	2.3	0.7	5
	4.4	3.39	2.4	0.7	6
f	0[5]	-	7.8	-	-
g	11.7	1.63	1.6	1.0	15
	8.0	2.40	1.8	0.8	-

[1] Individual results ; Tubocurarine = 100
[2] Dose in mg/kg i.v. producing 50 % neuromuscular blockade
[3] Dose in mg/kg i.v. producing 50 % vagal blockade
[4] Time in minutes for full recovery from onset of complete paralysis
[5] No block recorded at doses up to 8 mg/kg.

3d and **3f**, were inactive in doses up to 8 mg/kg. As expected from the spacial separation of its quaternary groups and their tri-*N*-methyl substituents, **3a** was depolarising, producing partial block and contracture of the chick biventer cervicis preparation (12) ; **3e** also showed similar properties. Only **3g** was non-depolarising as shown by the ready reversal of neuromuscular blockade by edrophonium. Both **3e** and **3g** showed low potencies compared to tubocurarine and both caused vagal blockade at doses below those required for neuromuscular paralysis. All three compounds, **3a**, **3e** and **3g**, were short-acting with a rapid rate of recovery.

EXPERIMENTAL

Unless stated otherwise, melting points were recorded on a Kofler Heizbank 184321 melting point apparatus. Infrared spectra were obtained on a Perkin-Elmer 157 instrument and were obtained on liquid films or as KCl discs (for solids). Proton magnetic resonance spectra were recorded on a Perkin-Elmer R12 instrument operating at 60 MHz. TMS was used as internal standard. IR and NMR data were obtained routinely and are available on request from the authors (RDW and GHD). Microanalytical results (C,H,N except where stated otherwise) were within ± 0.4 % of theoretical.

Quinol Diacrylate, **1a**. Acryloyl chloride (18.1 g ; 0.2 mole) in dry benzene (60 mL) was added over 0.5 h to quinol (11.0 g ; 0.1 mole) in benzene (100 mL) and triethylamine (21.0 g) stirred at 5° C. A further quantity of dry benzene (*ca* 100 mL) was added followed by triethylamine (10 mL) and the mixture stirred at 50° C for 0.5 h. The triethylamine hydrochloride was filtered off and the solvent removed *in vacuo* to leave a pale-red oil which solidified on addition of *n*-hexane (*ca* 80 mL). Re-crystallisation from *n*-hexane gave the diacrylate as colourless plates (10.1 g ; 46 %), m.p. 86° C (Lit (8), m.p. 86° C).

Catechol Diacrylate, **1b**. Triethylamine (21.0 g) in dry ether (60 mL) was added over 0.5 h to catechol (11.0 g ; 0.1 mole) and acryloyl chloride (18.1 g ; 0.2 mole) in dry ether (200 mL) stirred at 5° C. A further quantity of dry ether (100 mL) was added, followed by triethylamine (10 mL) and the mixture stirred at room temperature for a further 0.5 h. The triethylamine hydrochloride was filtered off and the solvent removed *in vacuo*. The red oil remaining was boiled with petroleum ether (b.p. 40-60° C ; 3 × 150 mL) and the separate decanted organic layers refrigerated to yield the *diacrylate* as pale-yellow needles (from petroleum ether, b.p. 40-60° C) (9.7 g ; 45 %), m.p. 35-36° C. *Anal.* for $C_{12}H_{10}O_4$: C,H.

Pyrogallol Triacrylate, **1f**. Pyrogallol (6.3 g ; 0.05 mole) was treated with acryloyl chloride (11.8 mL ; 0.15 mole) exactly as described for the synthesis of catechol diacrylate. The yellow solid produced on evaporation of the solvent was crystallised in petroleum ether (b.p. 60-80° C) to yield the *triacrylate* (5.2 g ; 36 %) as colourless needles, m.p. 80° C. *Anal.* for $C_{15}H_{12}O_6$: C,H.

1,2,3,4-Tetrahydro-6,7-dimethoxyisoquinoline was prepared as described previously (3). The hydrochloride had m.p. 256-257° C (Lit. (9), m.p. 253° C) and the free base m.p. 86° C (Lit. (10), m.p. 84-85° C).

Tetrahydropapaverine. Tetrahydropapaverine, as a colourless, viscous oil, was obtained as described previously (3, 4). The hydrochloride had m.p. 218° C (Lit. (11), m.p. 217-219° C).

1,4-Bis-(3-dimethylaminopropionyloxy)benzene, **2a**. Dimethylamine (1 mL, 33 % solution in ethanol) in dry ether (10 mL) was added over 15 min to a stirred solution of quinol diacrylate (0.5 g ; 0.0045 mole) in dry ether (10 mL) at 0° C. Stirring was continued at 0° C for 1 h and solvents removed *in vacuo*. The oil partially crystallised on standing. Crystallising solvents and other relevant data for this compound are given in Table II.

Bis-(3-aminopropionyloxy)benzene derivatives (**2b-e** and **2g**) and the *tris-amine*, **2f**. The following general method was adopted.

To the secondary amine (*ca* 2.0 g ; 2 mole equivalents ; see Table II) in dry ether or chloroform (*ca* 10 mL ; see Table II) at 0° C was added the appropriate bis-acrylate (1 mole equivalent) and the mixture stirred at room temperature for 2-48 h (Table II). The proton salts of **2b** and **2e** were prepared by addition of a saturated solution of hydrogen chloride in dry ether, followed by ether, and crystallised from ethanol or ethanol-ether. Data relating to amines **2b-g** are presented in Table II.

Quaternary salts (**3a** and **d-f**) were prepared by methods already described (3, 4 and 5). **3g** was prepared by adding the theoretical quantity of freshly distilled methylfluorosulphonate (" magic methyl " - care !) to a solution of pure **2g** in dry CH_2Cl_2 (approx. 20 % w/v solution). The solid which had precipitated after 0.5 h was filtered off, washed with CH_2Cl_2, then ether, and dried *in vacuo*. The microanalytical sample was obtained by dissolving the material in spectroscopically pure acetonitrile and adding the filtered solution dropwise

TABLE II. — *Phenolic bis-amine esters* (2).

Compound No.	Reaction solvent	Reaction time (hr)	% Yield of pure amine
2a	Et_2O	2	61[1]
b	Et_2O	6	99[2]
c	Et_2O	6	99[2]
d	$CHCl_3$	18	71[3]
e	$CHCl_3$	3	81[4]
f[6]	$CHCl_3$	2.5	69[5]
g	$CHCl_3$	48	69

[1] Yield refers to the free base after crystallisation from petroleum ether (b.p. 40-60°) and re-crystallisation twice from the same solvent. M.p. 38° C.

[2] NMR and tlc (Polygram/Alox N for tlc ; EtOH : EtOAc, 1 : 1) indicated purity of the product on evaporation of the solvent after the stated reaction time.

[3] After crystallisation of the dihydrochloride from EtOH (*see* also Table 3).

[4] The pure solid product, m.p. 150° C, was obtained by adding Et_2O (100 mL) to the reaction mixture, filtering off and drying *in vacuo*.

[5] After crystallisation of the trihydrochloride from ethanol (*see* also Table 3).

[6] Three mole equivalents of secondary amine employed in the synthesis (*see* experimental).

to mechanically stirred, filtered, dry ether (3, 4). Their characters are shown in Table III.

Neuromuscular blocking potency

Neuromuscular blocking potencies were determined on cat gastrocnemius muscle-sciatic nerve preparations as previously described (1). The results are given in Table I.

Vagal block

Vagal block was determined in anaesthetised cats as previously described (3). The results are given in Table I.

Acknowledgement. — We thank the U.K. Science Research Council for a research studentship to G. H. Dewar.

REFERENCES

(1) J. B. Stenlake, John Urwin, R. D. Waigh and R. Hughes, *Eur. J. Med. Chem.*, 1979, **14**, 77.
(2) J. B. Stenlake, John Urwin, R. D. Waigh and R. Hughes, *Eur. J. Med. Chem.*, 1979, **14**, 85.
(3) J. B. Stenlake, R. D. Waigh, John Urwin, G. H. Dewar, R. Hughes and D. J. Chapple, *Eur. J. Med. Chem.*, 1981, **16**, 508.
(4) J. B. Stenlake, R. D. Waigh, G. H. Dewar, R. Hughes, D. J. Chapple and G. G. Coker, *Eur. J. Med. Chem.*, 1981, **16**, 515.
(5) E. C. du Feu, F. J. McQuillin and R. Robinson, *J. Chem. Soc.*, 1937, 703.
(6) M. Tramontini, *Synthesis*, 1973, 703.
(7) R. W. Alder, *Chem. Ind.*, 1973, 983.
(8) F. Danusso, P. Ferroti and G. Ferroni, *Chimica Ind.*, Milano, 1967, **49**, 826.
(9) J. S. Buck, *J. Amer. Chem. Soc.*, 1934, **56**, 1769.
(10) J. M. Bobbitt, J. McNewkiely, K. L. Khanna and R. Ebermann, *J. Org. Chem.*, 1965, **30**, 2247.
(11) F. L. Pyman, *J. Chem. Soc.*, 1909, 1610.
(12) B. L. Ginsborg and T. Warriner, *Brit. J. Pharmacol. Chemother.*, 1960, **15**, 410.

TABLE III. — *Phenolic ester bis-amine hydrochlorides and quaternary salts* (3).

Compound No.	Melting point (°C) Hydrochlorides[1]	Quaternary salts[2]	Yield of quaternary salts from amine (3)	Microanalysis Formula	
3a	-	121-125	82[3]	$C_{18}H_{30}I_2N_2O_4$	C,H,N
b	171.5-172[4]	-	-	$C_{20}H_{34}Cl_2N_2O_4$	C,H,N
c	203-204[4]	-	-	$C_{20}H_{34}Cl_2N_2O_4$	C,H,N
d	199-202 (dec)	138-139	75[5]	$C_{36}H_{46}I_2N_2O_8, 0.5H_2O$	C,H,N
e	-	170-190	95[5]	$C_{36}H_{46}I_2N_2O_8$	C,H,N
f	161-166	146-150	88[5]	$C_{51}H_{66}I_3N_3O_{12}$	C,H,N
g	206 (dec.)[6]	Softens ca 150	43	$C_{54}H_{66}F_2N_2O_{18}S_2, 1H_2O$	C,H,N

[1] **3**, R = H, X⁻ = Cl.
[2] X⁻ as shown in Scheme 1.
[3] Quaternised in EtOH - MeI (1 : 1) and crystallised from EtOH - H_2O.
[4] Crystallised from EtOH - Et_2O.
[5] Quaternised (2, 3) in $CHCl_3$ and isolated by dripping the reaction mixture into Et_2O.
[6] *Anal.* for $C_{62}H_{62}Cl_2N_2O_{12}$, $2H_2O$: C, H, N.

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Biodegradable neuromuscular blocking agents

Part 6. — Stereochemical studies on atracurium and related polyalkylene di-esters

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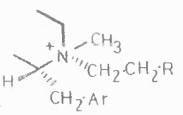
Summary. — Stereoisomers of atracurium (**3**, n = 5) and one of its homologues each having the configuration, *RR*, *SS* and *RS* at the C(1)-positions have been synthesised. The ratio of *cis* and *trans* components in the *RR*-, *SS*- and *RS*/*meso*-products, which contain three, three and four isomers respectively, measured by ^1H and ^{13}C NMR is approximately 3.0. The ratio of *cis-cis*-, *cis-trans*- and *trans-trans*-isomers in the isomer mixtures, determined by HPLC, is in reasonable agreement with the *cis*/*trans* ratios found by ^1H NMR. Variable temperature ^{13}C NMR suggests that the tetrahydropapaverinium ring system undergoes fast ring inversion at ambient temperature.
The relative molar neuromuscular blocking potencies of the three atracurium isomer products in anaesthetised cats were found to be *RR* (231), *RS*/*meso* (133) and *SS* (89) compared to that of tubocurarine (100). The significance of these relative potencies is discussed in relation to the potencies of norcoralydine and tubocurarine stereoisomers.

Résumé. — Des stéréoisomères d'atracurium (**3**, n = 5) et un de ses homologues, chacun ayant la configuration *RR*, *SS* et *RS* à la position C(1) ont été préparés par synthèse. Le rapport des constituants *cis* et *trans* dans les produits *RR*-, *SS*- et *RS*/*meso*, contenant trois, trois et quatre isomères respectivement, déterminé par RMN ^1H et ^{13}C est approximativement 3,0. Le rapport des isomères *cis-cis*-, *cis-trans*- et *trans-trans* dans les mélanges d'isomères, déterminés par chromatographie en phase liquide à haute performance, est en raisonnable accord avec les rapports donnés par la RMN ^1H. La RMN ^{13}C à température variable suggère que le système à anneau de tétrahydropapavérinium subit une inversion rapide à température ambiante.
Les puissances relatives de blocage neuromusculaire molaire des trois produits isomères d'atracurium dans les chats anesthésiés sont les suivantes : *RR* (231), *RS*/*meso* (133) et *SS* (89) par rapport à la tubocurarine (100). L'importance de ces puissances relatives est discutée par rapport aux puissances des stéréoisomères de norcoralydine et de tubocurarine.

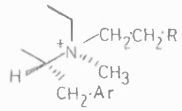
Zusammenfassung. — Es wurden Stereoisomere sowohl von Atracurium (**3**, n = 5) als auch eines seiner Homologen mit *RR*-, *SS*- und *RS*-Konfiguration an den C(1)-Positionen dargestellt. Das Verhältnis der *cis*- und *trans*- Komponenten der *RR*-(3 Isomere), *SS*-(3 Isomere) und *RS*/Meso-(4 Isomere) Verbindungen wurde durch ^1H und ^{13}C NMR Spektroskopie zu etwa 3.0 bestimmt. Das in den isomeren Mischungen durch HPLC bestimmte Verhältnis der *cis-cis*, *cis-trans*, und *trans-trans* Isomeren befindet sich in befriedigender Übereinstimmung mit den durch NMR-Spektroskopie bestimmten *cis-trans* Verhältnissen. Ergebnisse, die durch ^{13}C-NMR-Spektroskopie bei verschiedenen Temperaturen erhalten wurden, lassen vermuten, daß das Tetrahydropapaverinsystem bei Raumtemperatur schnelle Ringinversionen erleidet.
Die relative molare neuromuskuläre Blockierungskapazität der drei Atracurium-Isomeren wurde in anästhetisierten Katzen zu 231 (*RR*), 133 (*RS*/meso) und 89 (*SS*) bestimmt (Tubocurarin = 100). Die relativen Hemmkapazitäten der Stereoisomere wurden untereinander als auch mit denen von Norcoralydin und den Stereoisomeren von Tubocurarin verglichen und diskutiert.

Key-words : Atracurium — Polyalkylene di-esters — Neuromuscular blocking agents — Biodégradable agents — Stereochemical studies.

Atracurium (**3**, n = 5) and similar compounds in the related series of polyalkylene di-esters (**3**) show identical stereochemical features to laudexium (**1**). These compounds have four chiral centres at C(1) and N(2) in the two tetrahydropapaverine units. Because of molecular symmetry, the sixteen isomers which are theoretically possible reduce to ten. Atracurium and related quaternary salts, therefore, consist of four racemates and two *meso*-compounds. Each isomer can be defined by its configuration at C(1) relative to that of the tetrahydropapaverine unit (*R* or *S*) from which it is derived together with the relative configuration (*cis* **1** or *trans* **2**) about the two C_1—N_2 bonds. The *cis*-configuration (**1**) is defined arbitrarily as that in which the two bulky substituents [1-(3,4-dimethoxybenzyl) and 2-alkylene-ester groups] are *cis*.



R-cis-isomer (**1**)



R-trans-isomer (**2**)

Stereoisomeric quaternary salts having the configurations *RR*, *SS* and *RS* at the C_1 positions have been prepared

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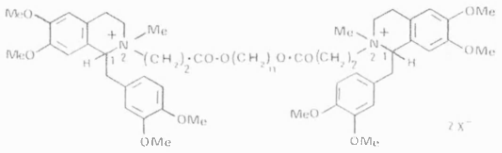
corresponding to the structures **3** (n = 2), and **3** (n = 5). In each case, the *RR*-, *SS*- and *RS*/*meso*-products are mixtures of three, three and four isomers respectively (Scheme 1). These products have not been separated into their component isomers. Their neuromuscular blocking potencies have been measured in anaesthetised cats, and correlated with estimates of their isomeric composition based on that of (±)-atracurium salts determined by NMR spectroscopy and HPLC.



(3)

Product	*Configuration of component isomers*			
	C_1	C_1—N_2	C_1	C_1—N_2
RR	R	cis	R	cis
	R	cis	R	trans
	R	trans	R	trans
SS	S	cis	S	cis
	S	cis	S	trans
	S	trans	S	trans
RS/meso	R	cis	S	cis
	R	trans	S	trans
	R	cis	S	trans
	R	trans	S	cis

Scheme 1.

CHEMISTRY

The *RR*- and *SS*- products **3** (n = 2 and 5) were prepared by methods already described (2, 3). Either *R*- or *S*-tetrahydropapaverine was condensed with the appropriate alkylene diacrylates (**4**) to give the corresponding optically active *NN'*-dioxa-dioxoalkylene-αω-bis-tetrahydropapaverine (as **6** but with *RR*- or *SS*-geometry). These optically active di-tertiary bases were quaternised with either methyl iodide (**3**, n = 2), methyl methanesulphonate (**3**, n = 2 and 5), or methyl benzenesulphonate (**3**, n = 5).

The *RS*/*meso* dimesylate (**7**, n = 5) was prepared as shown in Scheme 2. (*S*)-(−)-Tetrahydropapaverine was condensed with excess (5 mole) of pentamethylene diacrylate (**4**, n = 5) to yield the intermediate, *S*-(+)-1-tetrahydropapaverin-2′-yl-4,10-dioxa-3, 11-dioxotridec-12-ene (**5**). Condensation of the latter with *R*-(+)-tetrahydropapaverine gave the *RS* di-tertiary base, *NN'*-4,10-dioxa-3,11-dioxotridecylene-1-13-bis-tetrahydropapaverine (**6**) which was isolated as the oxalate salt. The base was quaternised with methyl methanesulphonate and methyl benzenesulphonate to yield the *RS*/*meso* dimesylate, and *RS*/*meso* dibesylate, **7** (n = 5) respectively. The *RS*/*meso*-dimesylate, **7** (n = 2), was prepared similarly starting from ethylene diacrylate and R-(+)-tetrahydropapaverine.

RESULTS AND DISCUSSION

Isomer composition

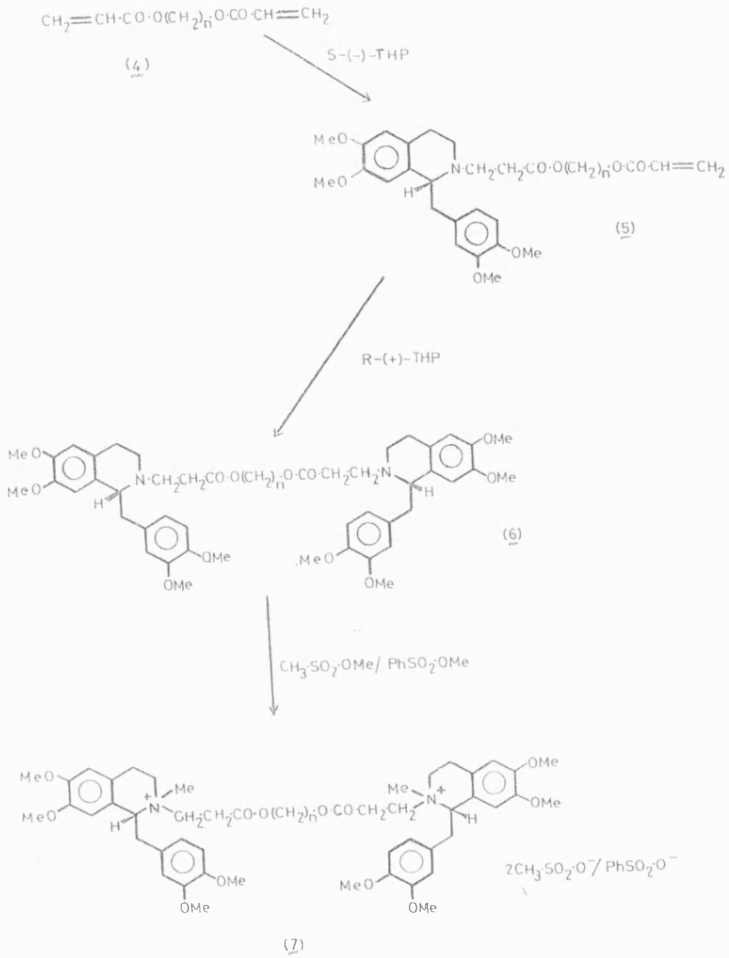
The NMR spectrum of *S*-*N*-ethyltetrahydropapaverinium methiodide shows splitting of the H-8 proton and $\overset{4}{N}CH_2CH_3$ signals (4) due to shielding differences in the *cis* and *trans* isomers. Recent ^1H studies have assigned the lower field H(8) proton signal at 5.79 δ to the *cis*-isomer and the higher field H(8) signal at 5.72 δ to the *trans*-isomer (5). Similar assignments have also been made for other related pairs of isomers with *N*-benzyl and *N*-butyl substituents (5), in conformity with a higher potential barrier to rotation in the *cis* configuration, and these conclusions have been confirmed by single crystal X-ray analysis of *trans*-*N*-3-hydroxypropyl-5′-methoxylaudanosinium perchlorate and *cis*-*N*-3-hydroxypropyl-5′-methoxylaudanosinium iodide monohydrate.

Atracurium salts (**3**, n = 5) and other compounds in the same series (**3**, n = 2) show a similar splitting of H-8 proton signals into two peaks with the major component at lower field (5.9 δ) and the minor component at 5.65 δ. Likewise, the ^{13}C NMR spectrum of Atracurium shows two *N*-Me resonances, at 47.4 ppm for the major component and 49.2 ppm for the minor component. Accordingly, the major component is assigned the *cis*-configuration in conformity with the conclusion of Lindon and Ferrige (6), determined by a combination of NMR nuclear Overhauser experiments and syntheses of laudanosine methiodide using ^{13}C-enriched methyl iodide, that direction of attack during quaternisation of 1-benzyl-*N*-methyl-tetrahydroisoquinolines is from the side of the ring opposite to the 1-benzyl side chain. The Atracurium assignments are confirmed by the similar *N*-Me resonances at higher field (46.8 ppm and lower field (49.0 ppm in *cis*- and *trans*-N-ethyltetrahydropapaverinium methiodide (5). The major atracurium components associated with the lower field H-8 signals at δ 5.9 are, therefore, *cis* and the corresponding minor components associated with the higher field H-8 signals at δ 5.65 are *trans*. Figure 1 shows the 400 MHz ^1H NMR spectrum of (±) Atracurium Besylate in dmso-d_6 solution.

The relative intensities of the two H-8 proton signals in (±)-atracurium besylate give a *cis*/*trans*-isomer ratio of *ca.* 3.0. The isomer ratio, however, cannot be determined precisely by routine 90 MHz NMR because of the near coincidence of the single H-8 resonance of laudanosine methiodide, which is present as a trace impurity, with the high field signal of atracurium. On the other hand, the ratio can be determined with much greater precision by high pressure liquid chromatography, which shows three separate peaks attributable to the *cis-cis*-, *cis-trans*- and *trans-trans*-isomers respectively (Fig. 2).

The overall ratio of *cis*- and *trans*- entities can be determined from the ratio of the area of the *cis-cis* peak + half of the area of the *cis-trans* peak to the area of the *trans-trans* peak + half of the area of the *cis-trans* peak.

Peaks are consistently observed by HPLC analysis of (±)-atracurium besylate (Fig. 2) for the *cis-cis*-, *cis-trans*-,



Scheme 2

trans-trans-isomers respectively within experimental error in the ratio of 10.5 : 6.2 : 1. From this, the calculated ratio of *cis*/*trans*-entities is 3.07 in reasonable agreement with the approximate ratio of 3.0 derived by 90 MHz NMR. Similar, though not precisely identical ratios, have been found for the mesylate salts.

High pressure liquid chromatography has also been used to determine the total isomer content and *cis-cis*, *cis-trans* and *trans-trans* isomer ratios of the *RR*-, *SS*- *RS*-/*meso*-isomer mixtures (Table II) and these are confirmed by the *cis*/*trans* ratios of 3.0, 3.0 and 3.2 obtained from the 90 MHz NMR spectra.

The areas of the individual *cis-cis*, *cis-trans* and *trans-trans* isomers can be predicted from the overall *cis-trans* ratio. The derivation, shown in Scheme 3, assumes that the two basic centres of the di-tertiary base are quaternised independently and form *cis* or *trans* centres in the ratio represented by the overall *cis*/*trans* ratio. Each *cis*-mono-quaternised molecule can then form a *cis-cis* atracurium molecule or a *cis-trans* molecule and again will do so in the ratio of the overall *cis-trans* ratio because this represents the probability of a *cis* or *trans* centre being formed on quaternisation of a tertiary base moiety. A similar argument applies for *trans*-monoquaternised molecules.

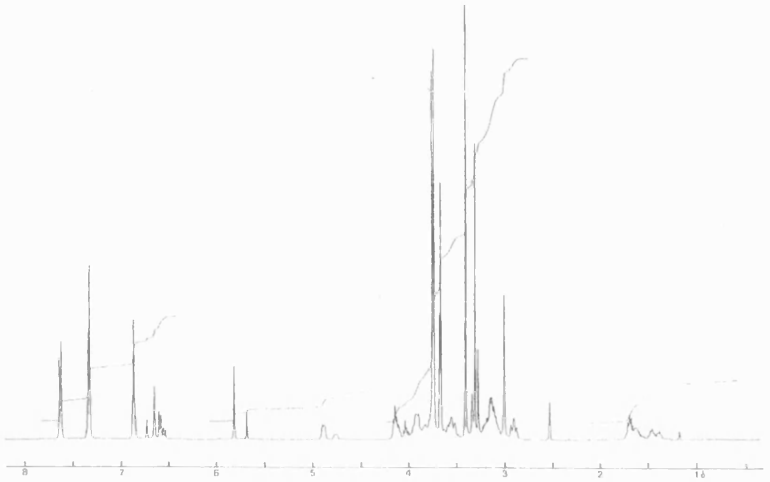


FIG. 1. — The 400 MHz ^1H NMR spectrum of atracurium dibesylate in dmso-d_6 solution.

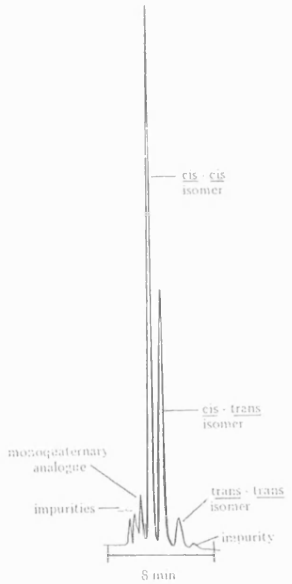
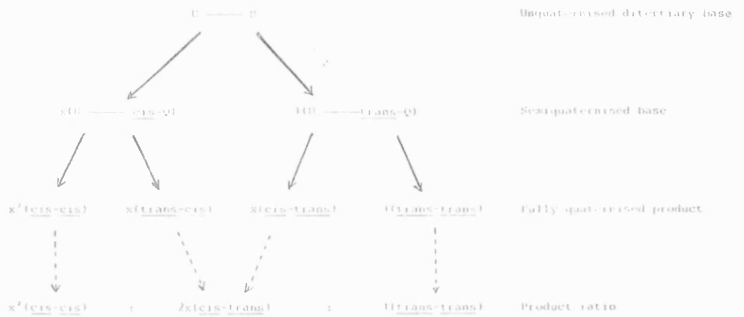


FIG. 2. — High-pressure liquid chromatogram of atracurium dibesylate separation. The various species are as labelled. Column : Partisil 10, 25 cm × 0.4 cm (i.d.) ; Mobile phase MeOH, HBr, H_3PO_4, 500:1:1 ; U.v. detection at 280 nm.

Hence it can be seen that the *cis-cis*, *cis-trans* and *trans-trans* isomers of Atracurium should occur in the ratio of $x^2 : 2x : 1$, where x is the overall *cis/trans* ratio. This prediction is consistent with observations made in all of the many preparations of (±)-atracurium besylate made to date.

Examination of the 400 MHz ^1H NMR spectra of a typical sample of (±)-atracurium besylate in $CDCl_3$ shows four *cis* peaks which are observed in the ratio 1 : 1 : 3 : 3 (Fig. 3 *a*). This conforms with the expectation that the two larger peaks arise from *cis-cis* molecules and the two smaller ones from the *cis*-residues in *cis-trans* molecules, since *RR*- and *SS*- forms, being mirror images, give identical NMR spectra, and the *RS*/*meso*-mixture will have the same relative abundance as the sum of the *RR*- and *SS*-forms. These conclusions are supported by the corresponding spectra for the *RR*- and *SS*- isomer mixtures each of which shows only two of the *cis* peaks in a 1 : 3 ratio corresponding to the *cis-trans* and *cis-cis* isomers respectively (Fig. 3 *b*). Theoretically, the *RS*/*meso* mixture should also show another pair of *cis* peaks in a 1 : 3 ratio, the first corresponding to the (*R*)-*cis*-(*S*)-*trans*/(*S*)-*cis*-(*R*)-*trans* mirror image pair and the second to the (*R*)-*cis*-(*S*)-*cis*-isomer. The isomer mixture obtained actually shows two pairs of peaks (Fig. 3 *c*), each in a 1 : 3 ratio with a pair to pair abundance ratio of 4 : 21 corresponding to a *RS*/*meso* content of 84 % together with some 16 % of *RR*- and *SS*-isomers probably arising from incomplete resolution of optically active tetrahydropapaverine used in the synthesis.

The corresponding *trans* resonances are less well resolved. The *trans-trans* and *trans-cis* resonances are not resolved at all in the spectra of *RR*- and *SS*- products. In the *RS*/*meso*-mixture the *trans-trans* resonance merely appears as a shoulder causing asymmetric broadening of the *trans-cis* peak, though this is observed more explicitly in (±)-atracurium where the *trans* resonances are clearly resolved.



Scheme 3 : To illustrate how the ratio of the three isomer mixtures of Atracurium can be derived from an overall *cis/trans* ratio. x is the overall *cis/trans* ratio.

FIG. 3. — The partial 400 MHz $_1$H NMR spectra of atracurium dibesylate isomers in $CDCl_3$ solution. Only the H8 region is shown. The top trace (a) is from a typical production batch of (±)—Atracurium Besylate, the centre trace (b) is from the *RR* isomer and the bottom trace (c) arises from the *RS/meso* material.

The arrows mark impurity peaks and the asterisks denote the presence of *RR* and *SS* isomers in the *RS/meso* material.

Conformational mobility

The question of conformational flexibility of atracurium is of considerable interest in relation to the relevance of stereo-selectivity as a criterion of drug-receptor interaction. Low temperature ^{13}C NMR studies of laudanosine base, salt and quaternised analogues (as **10**) over a range of temperature down to — 80° C have shed some light on the problem (5). In all these compounds, considerable broadening of the signals for the four α-carbons on nitrogen, C(4), the benzylic carbon and C-8 was observed in contrast to the remaining signals which remained sharp. It was concluded that these observations were in conformity with fast conformational changes at room temperature which are slowed by cooling with exchange broadening of signals which are substantially different in two contributing conformers.

Similar ^{13}C NMR studies have been conducted with (±)-Atracurium Besylate in which spectra have been recorded as a function of temperature. Acetone was chosen as solvent since by analogy with $(CD_3)_2SO$ it was expected to show minimal effects due to interaction between the two ends of the molecule, so that increased ambiguity from resolving *cis-cis*, *trans-cis* and *trans-trans* resonances would be minimised. Examples of the spectra are shown in Figure 4.

Integration of the ^{13}C resonances at 47.4 ppm and 49.2 ppm due to the *cis* and *trans* *N*-Me groups indicates a ratio of *ca* 3.0. The agreement with the value obtained by ^{1}H NMR from the study of the H-8 peaks is reasonable bearing in mind the known difficulties of integrating ^{13}C resonances.

All the ^{13}C resonances were sharp at 50° and 24° C. At — 19° C all aliphatic peaks remained sharp with the exception of the *trans* *N*-Me peak which had begun to broaden. At — 33° C the *trans* *N*-Me peak was broad and other aliphatic resonances began to broaden uniformly. At — 40° C all the aliphatic signals were broad but the *cis* *N*-Me remained sharp as did the besylate anion resonances and some of the atracurium aromatic signals.

At — 33° C some degrees of flexibility in the molecule such as the 1-dimethoxybenzyl rotation and aliphatic ester side chain movement, begin to slow down for both *cis-*

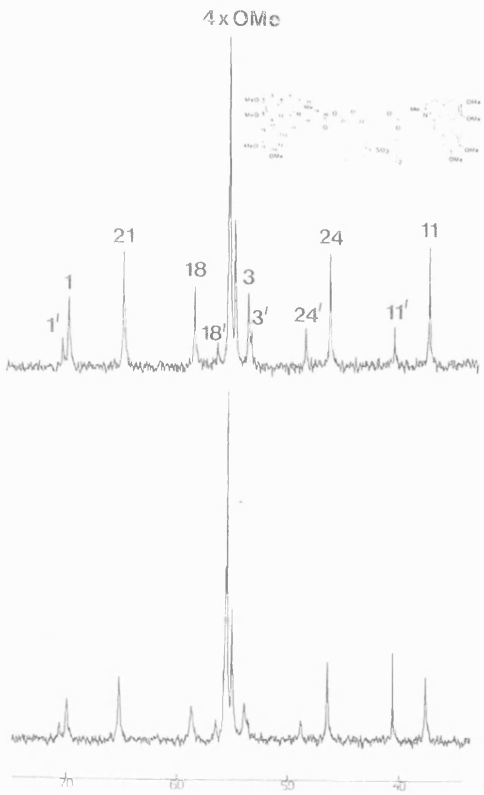


FIG. 4. — The partial 90.47 MHz $_{13}C$ NMR spectra of atracurium dibesylate at 254° K (top) and at 240° K (Bottom) showing differential broadening of the N-methyl (24), Benzyl (11) and N-CH_2 (18) resonances.

and *trans*-forms, thereby broadening all aliphatic resonances and obscuring any observation of ring inversion for the *cis*-entities. However, in contrast to the *N*-Me signals, the *cis* dimethoxybenzyl methylene peak broadens before the corresponding *trans* peak on cooling. This suggests that this methylene resonance is monitoring the benzyl group rotation which would be expected to slow down first in *cis* residues due to the greater steric hindrance in this isomer compared to that in the *trans* compound.

The observed line broadening of the *trans N* Me signal is consistent with the conclusion that the *trans* residues are undergoing a fast ring flip at ambient temperature between two conformations in which the energy difference is not large. This is also likely to be true of the *cis* form, where the energy difference between the two *cis* conformers is not expected to be large and indeed is likely to be less than of the *trans* isomer because it is probable that the $C_{ax} - N_{eq} \rightleftharpoons C_{eq} - N_{ax}$ inversion in the *cis* isomer requires less energy than the corresponding $C_{eq} - N_{eq} \rightleftharpoons C_{ax} - N_{ax}$ inversion of the *trans* isomer.

On balance, therefore the evidence favours fast ring inversion of both *cis* and *trans* forms at physiological temperatures (37° C). It must be emphasised, however, that observations which are fast on the NMR time scale occur in the 0.1-100 sec^{-1} range, which is extremely slow in terms of processes such as molecular tumbling or receptor binding. Hence, no firm conclusion on the relevance of ring flip to pharmacological effects in this series is possible. Equally there is no evidence to suggest that ring flip is a significant determinant in potency correlations between chemically related compounds or like three dimensional characteristics such as are discussed in the sequel.

Neuromuscular block

Neuromuscular blocking potencies were measured in anaesthetised cats by methods already described (2, 3). No significant differences were found between the potencies of iodide, methanesulphonate and benzenesulphonate salts of individual isomers. The results given in Tables I and II show that, as with the analogous laudexium isomers (1 ; tested on isolated chick biventer cervicis muscle), the (*RR*)-products of both compounds (**3**, n = 2 and 5) are significantly more potent than the corresponding (*SS*)- isomers. This difference is greater in the shorter chain laudexium methiodides (*RR*/*SS* potency ratio 2.75) and markedly more so in **3**, n = 2 (*RR*/*SS* potency ratio *ca* 4) than in the more potent atracurium derivatives, **3**, n = 5 (*RR*/*SS* potency ratio 2.5). Inadequacies in stereochemical fit are thus clearly compensated in the latter compounds by the ability of both charged centres to associate with the receptor effectively when the inter-quaternary distance is at its optimum. As anticipated the *RS*/*meso* isomer mixture is intermediate in potency between that of the (*RR*)- and (*SS*)-products.

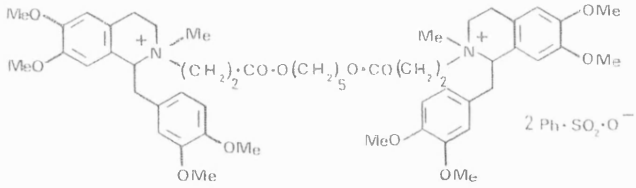
TABLE I. — *Neuromuscular blocking potencies of compound* **3** (n = 2) *isomeric products in anaesthetised cats.*

Isomeric product	PD 50 [1]
RR	0.42 ± 0.047 (5) [2]
SS	1.60 ± 0.24 [3] (3)

[1] Dose in mg/kg i.v. producing 50 % neuromuscular blockade ± SEM.
[2] Number of animals.
[3] Significantly different from that of *RR*-isomer $P < 0.05$

Observations that the (*RR*)-polyalkylene-bisquaternary salts of the atracurium series are more potent than the corresponding (*SS*)- products are consistent not only with the analogous laudexium isomers (1 ; tested on the isolated chick biventer cervicis muscle) but also with the greater potency of (*RR*)-isomers in the related *N*,*N*-decamethylene-bispavinium methiodides (7). These results, however, appear to be in conflict with those of the simple monoquaternary methiodide salts of laudanosine (4, 8), carnegine (8), *N*-methylpavine (8), corydine (8), isocorydine (8), glaucine (8)

TABLE 2. — *Atracurium stereoisomers. Isomer composition and mean neuromuscular blocking potencies in groups of 4 anaesthetised cats.*



Product	*Configuration of component isomers*				*Isomer content*	*Isomer ratios*	*Percent cis and trans components*				PD_{50}[1]	*Relative molar potency*[3]
	C_1	C_1—N_2	C_1	C_1—N_2	(HPLC assay)	(HPLC)	*R—cis*	*R—trans*	*S—cis*	*S—trans*		(Calc. to 100 % isomer content)
RR	R	cis	R	cis		10.7	76.5	23.5	0	0	0.103 ± 0.008	231
	R	cis	R	trans	88.0	6.5						
	R	trans	R	trans		1.0						
RS/meso	R	cis	S	cis		10.3	38.2	11.8	38.2	11.8	0.179 ± 0.015[2]	133
	R	cis	S	trans	88.2							
	R	trans	S	cis		6.3						
	R	trans	S	trans		1.0						
SS	S	cis	S	cis		10.6	0	0	76.5	23.5	0.25 ± 0.033[2]	89
	S	cis	S	trans	93.6	6.6						
	S	trans	S	trans		1.0						

[1] Dose in mg/kg i.v. producing 50 % neuromuscular blockade ± SEM.
[2] Significantly different from that of *RR*-isomer $P < 0.01$.
[3] Tubocurarine = 100.

and boldine (8) in which the (*S*)- series isomers are all more potent in the cat than their corresponding (*R*)- isomers.

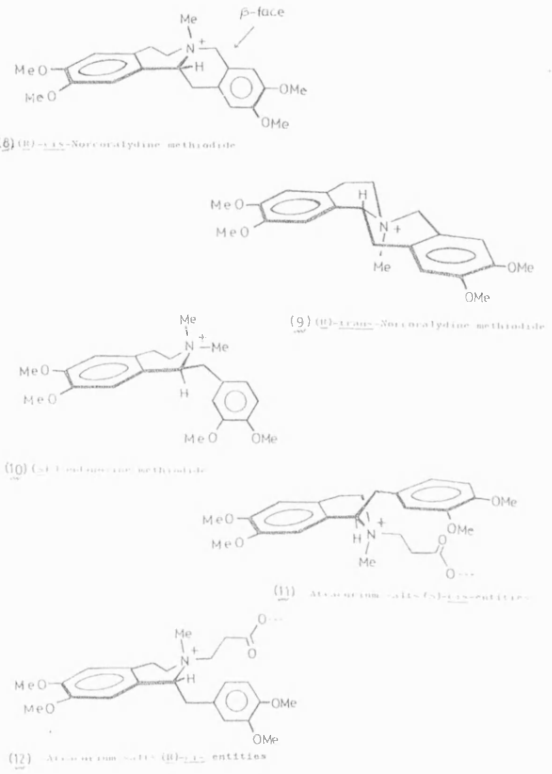
In contrast to these monoquaternary salts, the potencies of the four stereoisomers of the closely related norcoralydine methiodides are in the order (R)-*cis* > (R)-*trans* > (S)-*trans* > (S)-*cis* (9). This potency order is consistent with ion-pair bonding to an anionic receptor site which is stereoselective for the quaternary centre and its environs at the upper, β-face, of the molecule as represented in **8**. This hypothesis has been tested, and finds support in the enhanced potencies observed in (*R*)-*cis*-norcoralydine trideuteriomethiodide, (*R*)-*cis*-[8,8-^{2}H]-norcoralydine methiodide, (*R*)-*cis*-[8,8-^{2}H]-norcoralydine trideuteriomethiodide and (*R*)-*cis*-[13, 13, 13^a-^{2}H]-norcoralydine trideuteriomethiodide (10) compared to that of (*R*)-*cis*-norcoralydine methiodide. These compounds provide a closer fit to the receptor and hence show higher potency than the corresponding undeuterated compound due to the lower zero point energy of deuterium. This reduces the amplitude of vibration of the C—D bonds compared to the corresponding C—H bonds, and shortens the van der Waals radius in deuterium compared to hydrogen.

Circular dichroism spectrometry also suggests that (*S*)-laudanosine methiodide favours a combination of ring-conformation and C(1)-substituent orientation that offers the same β-face receptor recognition features as (*R*)-*cis*-norcoralydine methiodide (**8**), the most potent of the four stereoisomeric norcoralydine methiodides (9). It has also been established (11) that isotubocurarine (12) which is some 2.3 times more potent than tubocurarine, exhibits the same receptor-favourable, β-face, stereochemical characteristics about its sole quaternary centre as (*R*)-*cis*-norcoralydine methiodide. This feature is in contrast to that in tubocurarine in which the β-face R-C(1)—N geometry and ring conformation about the single quaternary centre relates to the pharmacologically less active (*S*)-*trans*-norcoralydine methiodide (**9**).

NMR and HPLC evidence, although not completely unequivocal, now implies that the major components of (**3**, n = 2 and 5), and presumably, those of laudexium are *cis*-entities (**12**) in which pseudo-equatorial and pseudo-axial orientations, or their converse, are favoured for the bulky *N*-substituent and C(1)-dimethoxybenzyl substituents respectively.

Examination of models shows that only the (*R*)-*cis* entities of compounds **3** (n = 2 and 5) in their C_{ax}—N_{eq} conformations have both the same substituent configurations and ring conformation, as (*R*)-*cis*-norcoralydine methiodide. Thus the most active of the Atracurium isomer products consists predominantly of (*R*)-*cis*-components which correspond closely in molecular shape to that of (*R*)-*cis*-norcoralydine methiodide, which in turn offers the most favourable β-face receptor recognition features of the four norcoralydine isomers for the induction of neuromuscular blockade (9, 10 and 11). Similarly, (*R*)-*trans*, (*S*)-*cis*- and (*S*)-*trans*-entities in **3** (n = 2 and 5) feature ring conformations and substituent orientations which relate to those of the corresponding norcoralydine methiodide stereoisomers, all of which are less potent than (*R*)-*cis*-norcoralydine methiodide. The relative contributions of the four stereochemical entities to potency may, therefore, be expected to be in the same relative order in both series.

The higher potency of the (*RR*)-products is, therefore, consistent with their high content (*ca* 75 %) of (*R*)-*cis* entities (**12**) with their capability of adopting the more favourable neuromuscular blocking stereochemical features



Scheme 4.

of (*R*)-*cis*-norcoralydine methiodide (**8**). Likewise, the less potent (*SS*)-products consist mainly (*ca* 75 %) of entities which can only relate to the stereochemical parameters of the pharmacologically less favourable (*S*)-*cis*-norcoralydine methiodide (**11**). The *RS*/*meso*-products with about 37.5 % each of favourable (*R*)-*cis*- and less favourable (*S*)-*cis*-entities should, therefore, be intermediate in potency as found (1) in the laudexium series (relative potencies of *RR*, *RS*/*meso*, *SS*, 275/140/98 ; tubocurarine = 100). A similar potency order (*RR*, *RS*/*meso*, *SS*, 231/133/89 seems to be apparent in **3**, n = 5 (Table II) with the *RS*/*meso*-product intermediate between *RR* and *SS*.

EXPERIMENTAL

Unless stated otherwise, melting points were recorded on a Kofler Heizbank 184321 melting point apparatus. Infrared spectra were obtained on a Perkin-Elmer 157 instrument using either liquid films or KCl discs (for solids). Routine proton magnetic resonance spectra were recorded for synthetic work on a Perkin-Elmer R32 instrument operating at 90 MHz. TMS was used as internal standard. IR and NMR data were in accord with the structures given. Microanalytical results (C, H, N except were stated otherwise) were within ± 0.4 % of theoretical. Thin-layer chromatography was run on Polygram Sil G/UV 154.

N, N'-*4,7-Dioxa-3,8-dioxodecylene-1, 10-diyl-bis-(R)-(—)-tetrahydropapaverine* was prepared from (*R*)-(+)-tetrahydropapaverine (1.0 g) and ethylene diacrylate (0.26 g) by the method described for the corresponding (+)-compound (2). The *dioxalate* was obtained as a colourless solid from ethanol (1.02 g ; 66 %), m.p. 151-153° C. *Anal.* for $C_{52}H_{64}N_2O_{20}$, H_2O : C, H, N. The *dihydrochloride* was obtained as an almost colourless solid from isopropanol, m.p. 125-132° C $[\alpha]_D^{20}$ — 57.07° (*c*, 1.104 in $CHCl_3$).

The dioxalate (1.01 g) was dissolved in water, basified with saturated sodium hydrogen carbonate solution, and the precipitated base extracted with chloroform. The combined extracts were dried (Na_2SO_4 anhydrous) and the solvent evaporated *in vacuo* to yield N,N'-*4,7-Dioxa-3,8-dioxodecylene-1,10-diyl-bis-(R)-(—)-tetrahydropapaverine* as a colourless solid (0.85 g ; 69 %), m.p. 47-49° C $[\alpha]_D^{20}$ — 58.2° (*c*, 1.323 in $CHCl_3$). R_F 0.55 in ethyl acetate : ethanol (1 : 1).

N-N'-*4,7-Dioxa-3,8-dioxodecylene-1,10-diyl-bis-(S)-(+)-tetrahydropapaverine* was prepared from (*S*)-(—)-tetrahydropapaverine as described for the bis-(*R*)-isomeric product. The *dioxalate* had m.p. 151-153° C. Anal. for $C_{52}H_{64}N_2O_{20}$, H_2O : C, H, N. The dihydrochloride had m.p. 124-132° C $[\alpha]_D^{20}$ + 58.12° C (*c*, 1.253 in $CHCl_3$). The *base* was obtained as a colourless solid m.p. 48-50° $[\alpha]_D^{21.5}$ + 58.9° (*c*, 1.021 in $CHCl_3$). R_F 0.55 in ethyl acetate : ethanol (1 : 1).

N-N'-*4,10-Dioxa-3,11-dioxotridecylene-1,13-diyl-bis-(R)-(—)-tetrahydropapaverine*. (*R*)-(+)-Tetrahydropapaverine (3.39 g) and 1,5-pentamethylenediacrylate (1.0 g) were heated with glacial acetic acid (0.2 mL) at 70° C for 4 hr. The reaction mixture was dissolved in toluene, the solution stirred for 5 hr with silica gel 60 (Merck, column chromatography grade, 70-230 mesh), filtered and evaporated to give a light brown viscous oil. The product in dry acetone (50 mL) treated with a solution of oxalic acid in dry acetone (6 %) yielded N,N'-*4,10-dioxa-3,11-dioxotridecylene-1,13-bis-(R)-(—)-tetrahydropapaverine dioxalate* as a colourless crystalline solid (78 %), m.p. 119-122° C $[\alpha]_D^{21}$ — 62.8° (*c*, 1.051 in $CHCl_3$) $[\alpha]_D^{15}$ — 43.28° (*c*, 2.087 in water). Anal. for $C_{55}H_{70}N_2O_{20}$: C, H, N. The *base* was obtained as a colourless oil $[\alpha]_D^{22.5}$ — 53.62° (*c*, 1.408 in $CHCl_3$). R_F 0.54 in ethyl acetate : ethanol.

N, N'-*4,10-Dioxa-3, 11-dioxotridecylene-1,13-diyl-bis-(S)-(+)-tetrahydropapaverine* was prepared from (*S*)-(—)-tetrahydropapaverine (4.14 g) and 1,5-pentamethylene diacrylate (1.22 g) by the method described for the bis-(*R*)-isomeric product. The *dioxalate* was obtained as a colourless crystalline solid (76 %), m.p. 116-119° C $[\alpha]_D^{21}$ + 62.1° (*c*, 1.035 in $CHCl_3$), $[\alpha]_D^{22}$ + 44.78° (*c*, 1.155 in water). *Anal.* for $C_{55}H_{70}N_2O_{20}$: C, H, N. The *base* was obtained as a colourless oil $[\alpha]_D^{23}$ + 52.65° (*c*, 0.961 in $CHCl_3$). R_F 0.54 in ethyl acetate : ethanol (1 : 1).

(R)-(—)-1-Tetrahydropapaverin-2'-yl-4,7-dioxa-3,8-dioxodec-9-ene (**5**, n = 2). (*R*)-(+)-Tetrahydropapaverine (0.92 g) in dry benzene (20 mL) was added dropwise to ethylene diacrylate (2.62 g) in dry benzene (10 mL), and the mixture refluxed for 4 hr. The solvent was evaporated *in vacuo*, and the oily residue washed three times with light petroleum (b.p. 40-60° C ; 100 ml). The oily residue was dissolved in benzene (10 mL) and light petroleum added to precipitate an oil. Re-solution in benzene and reprecipitation with light petroleum twice more gave an oily mass, which solidified on drying over P_2O_5 *in vacuo* at 50° C, to yield (*R*)-(—)-1-*tetrahydropapaverin-2'-yl*-4,-7-*dioxa-3,8-dioxodec-9-ene* (0.61 g ; 44 %), m.p. 52-55° C, $[\alpha]_D^{23}$ — 44.2° (*c*, 0.625 in $CHCl_3$).

(S)-(+)-1-Tetrahydropapaverin-2'-yl-4,10-dioxa-3,11-dioxotridec-12-ene (**5**, n = 5). (*S*)-(—)-Tetrahydropapaverine (3.01 g) and 1,5-pentamethylene diacrylate (9.34 g) were heated with glacial acetic acid (0.2 mL) at 70° C for 4 hr. The product was dissolved in ether (400 mL) and shaken with dilute hydrochloric acid. The aqueous layer was washed twice with ether (200 mL and 100 mL), made alkaline with potassium hydroxide solution and extracted with ether. The ether solution was dried (anhydrous Na_2SO_4) and evaporated to yield an oily residue (4.34 g ; 89 %), which showed three spots of R_F 0.75, 0.50 and 0.37 when chromatographed on thin layer using Polygram sil G/UV_{254} and chloroform/ethanol (95 : 5).

The components of the mixture were separated on a column (360 mm × 22 mm) of silica gel 60 (Merck, 70-230 mesh) using chloroform followed by chloroform/ethanol (99 :1). Evaporation of the eluate gave (*S*)-(+)-1-*tetrahydropapaverin-2'-yl-4,10-dioxa-3*, 11-dioxotridec-12-ene (3.47 g ; 71 %), $[\alpha]_D^{19.5}$ + 38.50° (*c*, 1.196 in $CHCl_3$), V_{max} : 1730 cm^{-1} (ester C = O) ; 1640 (— CH = CH_2). R_F 0.50 on Polygram sil G/UV_{254} in chloroform/ethanol (95 :5). Homogeneity was confirmed by HPLC using a Partisil 10 μm silica column and solvent system acetonitrile/water/phosphoric acid (90 : 9 :1) as described under High Pressure Liquid Chromatography.

*(RS)/meso-*N,N'-*4,7-Dioxa-3,8-dioxodecylene-1,10-diyl-bis-tetrahydropapaverine* (**6**, n = 2). (*R*)-(—)-1-Tetrahydropapaverine-2'-yl-4,7-dioxa-3,8-dioxodec-9-ene (0.33 g) and (*S*)-(—)-tetrahydropapaverine (0.215 g) were refluxed in dry benzene (15 mL) for 48 hr with constant stirring. The solvent was evaporated, the residue dissolved in chloroform (3 mL), and the solution treated with a saturated solution of oxalic acid in dry ether. The white flocculent precipitate was recrystallized from ethanol to yield *(RS)/meso*-N,N'-*4,7-dioxa-3,8-dioxodecylene-1,10-bis-tetrahydropapaverine dioxalate* (0.40 g ; 65 %), m.p. 87-90° C. Mixed m.p. with (+) base oxalate 85-130° C, $[\alpha]_D^{22.0}$ ± 0°, R_F 0.55 in ethyl acetate : ethanol (1 :1). *Anal.* for $C_{52}H_{64}N_2O_{20}$, H_2O : C, H, N. The *base* (**6**, n = 2) was obtained as a gummy solid.

(RS)/meso N, N'-*4,10-Dioxa-3,11-dioxotridecylene-1,13-diyl-bis-tetrahydropapaverine* (**6**, n = 5) prepared from (*S*)-(+)-1-tetrahydropapaverin-2'-yl-4,10-dioxa-3,11-dioxotridec-12-ene (3.17 g) and (*R*)-(+)-tetrahydropapaverine (1.98 g) by the method described for the preparation of *(RS)/meso-N,N'*-4,7-dioxa-3,8-dioxodecylene-1,10-bis-tetrahydropapaverine. The *dioxalate* was obtained as colourless crystals (3.78 g, 74 %), m.p. 107-110° C, $[\alpha]_D^{21}$ ± 0°. *Anal.* for $C_{55}H_{70}N_2O_{20}$: C, H, N. The *base* (**6**, n = 5) was obtained as an oil, $[\alpha]_D^{19.0}$ ± 0° (*c*, 1.018 in $CHCl_3$), R_F 0.54 in ethyl acetate : EtOH (1 :1).

Quaternary salts

The *(RR)*-, *(SS)*- and *(RS)/meso*-di-tertiary bases (**6**, n = 2 and 5) were quaternised with either methyl iodide, methyl methanesulphonate or methyl benzenesulphonate by methods as previously described (3). The characteristics of the product isomers are described in Table 3.

Isomer content and ratios of atracurium besylate isomer products

Isomer content and ratios were determined by high performance liquid chromatography. The apparatus used was constructed from a CE 210 coil pump fitted with an injection port (Cecil Instruments Ltd.) and a chromatographic column 250 mm × 4 mm i.d. precision bore, seamless stainless-steel tubing. All connections were made with low dead-volume stainless steel couplings (Reeve Angel Scientific Ltd.). Injections were made with a 10 μl Type 701N syringe (Hamilton Micromeasure BV) through a silicone rubber septum faced with PTFE and the column eluate monitored using a CE 212 variable-wavelength ultraviolet spectrophotometer fitted with a 10 μl flow cell (Cecil Instruments Ltd.). Results were displayed on a Autograph S pen recorder (Shandon Southern Instruments Ltd.) and peak areas were integrated electronically with an Autolab System IVb Chromatography Data Analyzer (Spectra-Physics Ltd.).

The column was slurry packed at a pressure of 35 MNm^{-2} with 10 μm particle size silica gel. Two systems were used under the conditions set out below. System 1 resolves atracurium from its impurities and was used for the determination of isomer content. System 2 resolves the isomers and was used to determine the isomer ratios. Isomer content and isomer ratios of the atracurium besylate isomer products are given in Table 2.

System 1. Mobile phase, acetonitrile : water : phosphoric acid (90 : 9 : 1). Flow rate, 2 mL min^{-1}. Loading, 5 L of 1 % w/v aqueous solution. Detection at 280 nm. Sensitivity, 0.2 AUFS.

System 2. Mobile phase, methanol : phosphoric acid : hydrobromic acid (500 : 1 : 1). Flow rate, 1 mL min^{-1}. Loading, 2 L of a 1 % aqueous solution. Detection at 235 nm. Sensitivity, 0.1 AUFS.

NMR spectroscopy

Cis/trans ratios were determined routinely using 90 MHz ^1H NMR of dmso-d_6 solutions. The spectra were acquired with a Bruker HFX-90 instrument coupled to an Instem DATAMAG data system, operating in the pulse-FT mode at 24° C. Sample concentration was routinely 50 mg . mL^{-1}. Detailed studies of the *RR*, *SS* and *RS/meso* mixtures and their *cis/cis*, *cis/trans* and *trans/trans* components were performed using ^1H NMR at 400 MHz in $CDCl_3$ solution on a Bruker WM-400 instrument at ambient temperature.

The conformational mobility of atracurium was probed by the measurement of ^{13}C NMR spectra at 90.57 MHz on a Bruker WM-360 spectrometer using an acetone-d_6 solution containing a small amount of $CDCl_3$. Measurements were taken at 323°, 297°, 254°, 240° and 233° K.

Neuromuscular blocking potencies

Neuromuscular blocking potencies were determined on gastrocnemius muscle-sciatic nerve preparations from cats as previously described (13). The results are given in Tables 1 and 2.

TABLE 3.

Compound	Isomeric product	Salt	m.p. °C	Specific rotation ($CHCl_3$)	Anal.
3 (n = 2)	RR	I^-	122-125	$[\alpha]_D^{20}$ — 48.9° (c,1.208)	C,H,N
	SS	I^-	122-126	$[\alpha]_D^{20}$ — 48.1° (c,1.105)	C,H,N
	RR	$CH_3{\cdot}SO_2{\cdot}O^-$	105-113	$[\alpha]_D^{23.5}$ — 55.9° (c,0.948)	C,H,N
	SS	$CH_3{\cdot}SO_2{\cdot}O^-$	105-114	$[\alpha]_D^{21.5}$ + 56.4° (c,1.140)	C,H,N
7 (n = 2)	RS/meso	$CH_3{\cdot}SO_2{\cdot}O^-$	100-112	$[\alpha]_D^{21}$ ± 0° (c,0.049)	C,H,N
3 (n = 5)	RR	$CH_3{\cdot}SO_2{\cdot}O^-$	110-114	$[\alpha]_D^{24.5}$ — 41.7° (c,1.323)	C,H,N
	SS	$CH_3{\cdot}SO_2{\cdot}O^-$	110-114	$[\alpha]_D^{24}$ — 40.3° (c,1.016)	C,H,N
7 (n = 5)	RS/meso	$CH_3{\cdot}SO_2{\cdot}O^-$	102-107	$[\alpha]_D^{24}$ ± 0° (c,0.935)	C,H,N
3 (n = 5)	RR	Ph $SO_2{\cdot}O^-$	*	$[\alpha]_D$ — 44.0° (c,1.000)	C,H,N
	SS	Ph $SO_2{\cdot}O^-$	*	$[\alpha]_D$ + 30.0° (c,1.000)	C,H,N
7 (n = 5)	RS/meso	Ph $SO_2{\cdot}O^-$	*	$[\alpha]_D$ ± 0° (c,1.000)	C,H,N

* These samples did not give definite melting points.

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REFERENCES

(1) J. B. Stenlake, W. D. Williams, N. C. Dhar and I. G. Marshall, *Eur. J. Med. Chem.*, 1974, **9**, 239.

(2) J. B. Stenlake, R. D. Waigh, J. Urwin, G. H. Dewar and Roy Hughes, *Eur. J. Med. Chem.*, 1980, **16**, 508.
(3) J. B. Stenlake, R. D. Waigh, G. H. Dewar, Roy Hughes, D. J. Chapple and G. G. Coker, *Eur. J. Med. Chem.*, 1981, **16**, 515.
(4) J. B. Stenlake, W. D. Williams, N. C. Dhar and I. G. Marshall, *Eur. J. Med. Chem.*, 1974, **9**, 233.
(5) H. A. El-Sayad, R. A. Swaringen, D. A. Yeowell, R. C. Crouch, S. Hurlbert, R. W. Miller and A. T. McPhail, *J. Chem. Soc.*, Perkin Trans., I, 1982, 2067.
(6) J. C. Lindon and A. G. Ferrige, *Tetrahedron*, 1980, **36**, 2157.
(7) A. A. Genenah, T. O. Soine and N. A. Shaath, *J. Pharm. Sci.*, 1975, **64**, 62.
(8) P. W. Erhardt and T. O. Soine, *ibid.*, 1975, **64**, 53.
(9) J. B. Stenlake, W. D. Williams, N. C. Dhar, R. D. Waigh and I. G. Marshall, *Eur. J. Med. Chem.*, 1974, **9**, 243.
(10) J. B. Stenlake and N. C. Dhar, *Eur. J. Med. Chem.*, 1978, **13**, 343.
(11) J. B. Stenlake, *Molecular Interactions at the Cholinergic Receptor in Neuromuscular Blockade*. Progress in Medicinal Chemistry. Volume 16. Eds. G. P. Ellis and G. B. West, Elsevier/North-Holland Biomedical Press, 1979, 257.
(12) T. O. Soine and J. Naghaway, *J. Pharm. Sci.*, 1975, **63**, 1643.
(13) R. Hughes and D. J. Chapple, *Brit. J. Anaesth.*, 1976, **48**, 59.

The chemistry behind atracurium

Roger D. Waigh

Muscle relaxants are used in surgery to make the surgeon's job easier and the operation safer. When muscles are relaxed, particularly in the abdomen, much lower doses of anaesthetic are needed—just enough to produce unconsciousness. Before these drugs were available the anaesthetic had also to serve as a muscle relaxant, which meant that the required high doses produced a deep and dangerous depression of the central nervous system. Atracurium is a new muscle relaxant and is one of the very few drugs designed and synthesised in an academic environment.

All the muscle relaxants used in surgery stem from the original observation of the paralysing South American arrow poison, curare, which contains a mixture of alkaloids, the best known being tubocurarine (*1*). Like the arrow poison, the modern drugs paralyse the diaphragm and cause death by suffocation in the absence of artificial respiration. There would therefore be a major practical advantage in using a muscle relaxant that does not persist in the body beyond the end of the operation, so that the patient can be returned to the ward breathing normally. Several workers have searched for the ideal drug.[1]

In the 1960s, research groups in the department of pharmacy at the University of Strathclyde and the chemistry department at Glasgow University had combined to find the structure of an alkaloid[2] from the Egyptian plant *Leontice leontopetalum*, itself of no great medicinal importance. Isolated as the reineckate salt, the quaternary alkaloid hydroxide was prepared by passing the compound down an ion exchange column. The researchers were concerned to find that evaporation of the solution thus obtained changed the properties of the material very considerably: there was a much stronger ultraviolet absorption and no quaternary nitrogen. These observations were explained[2] as the base catalysed Hofmann elimination of the alkaloid, now known as petaline(*2*), with anchimeric (neighbouring group) assistance from the nearby 8-hydroxy group.

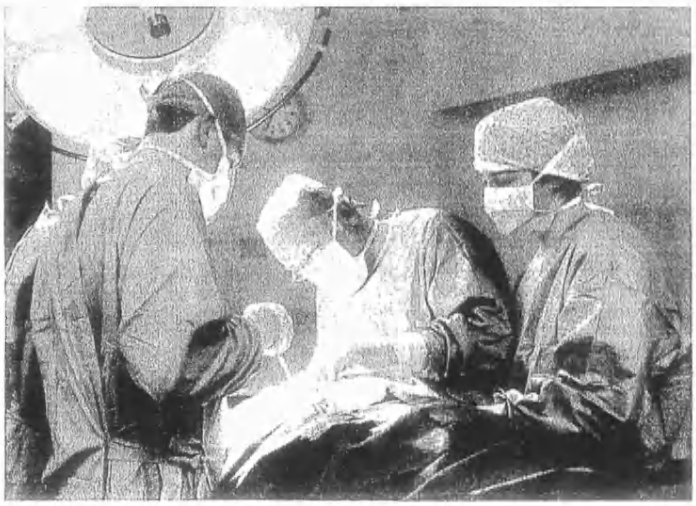
The Strathclyde group had been active for many years in the search for better muscle relaxants, so by putting two and two together they came up with the idea that a base catalysed Hofmann elimination of a compound with two petaline units might produce a muscle relaxant with a shorter duration of action, allowing the patient to be returned to the ward more quickly. To explain why this might work a little pharmacology is required; more details are available elsewhere.[1]

Pharmacologists have shown that muscles are controlled by nerve impulses, which are transmitted over the nerve–muscle junction by the release of minute amounts of acetylcholine (*3*), itself a quaternary ammonium salt. Other quaternary salts—such as the arrow poison alkaloids—can interfere with this process, and the interruption of the flow of chemical messages causes the muscle to relax. It has also been found that compounds with two quaternary nitrogens were often more potent inhibitors, provided that the nitrogens were separated from each other—the best distance apart was thought to correspond to 10 methylene units, as in the potent but long acting drug laudexium (*4*). Furthermore, it was almost invariably true that tertiary amines were inactive or much less active than the corresponding quaternary salts. The logic was compelling: the combination of two 'end groups' as in (*2*) into a bis-quaternary compound like (*4*) might give a compound which would decompose in alkaline solution to give an inactive bis-tertiary base. Blood and most body tissues are kept at pH 7.4 by sensitive biochemical mechanisms—would this pH be sufficient to promote Hofmann elimination? The Medical Research Council (MRC) was sufficiently intrigued to fund a research fellowship for three years, and I took up the post at Strathclyde in October 1969.

The answer to the main question—was pH 7.4 sufficient to promote elimination in a quaternary benzylisoquinoline—was answered even before the fellowship started. Further work at Glasgow had shown that compounds similar to (*4*) also underwent Hofmann elimination when the quaternary hydroxide was evaporated, so there was nothing special about petaline, after all. The 8-hydroxy group in (*2*) was not required to promote elimination and there was little point in synthesising a bis-quaternary analogue, because there was no indication that such a compound would decompose more rapidly than (*4*). The project had collapsed even before it had begun.

The next few weeks were taken up with an intensive library search. A

Muscle relaxants—a valuable aid in modern surgery. (Photo: SPL)

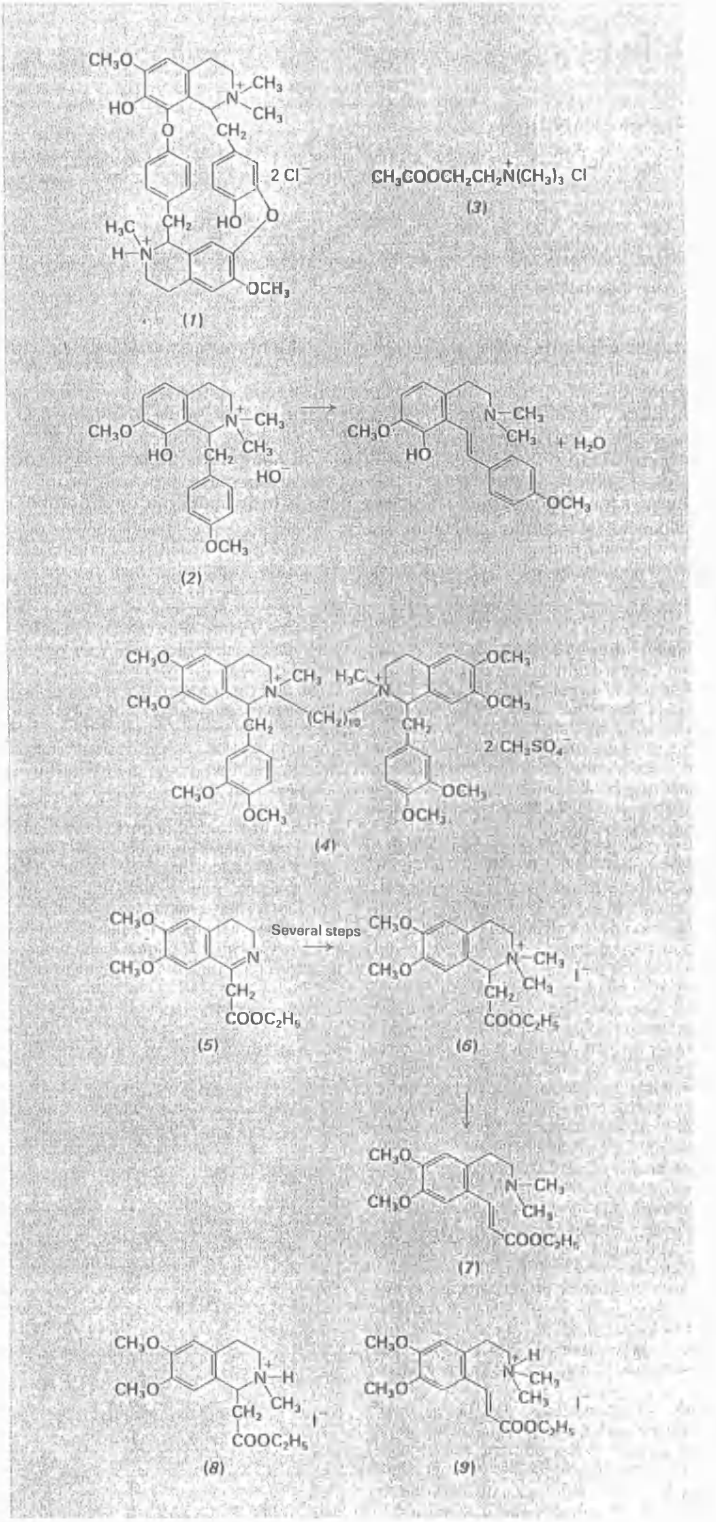


glimmer of light came from the reported rapid decomposition in alkali of the tetrahydroisoquinolinium salt (*6*),[3] obtained in a few steps from the dihydroisoquinoline (*5*), itself the product of the Bischler–Napieralski cyclisation of a malonamide. The decomposition of (*6*) was aided at the rate determining step by the carbonyl group adjacent to the reactive centre and was several orders of magnitude faster than the breakdown of petaline (*2*). It was possible to record the ultraviolet spectrum of (*6*), to add one drop of very dilute alkali to the cuvette, and immediately to record the spectrum of the base (*7*). The very existence of (*6*) was crucial, because it showed that compounds which underwent rapid Hofmann elimination in very dilute alkali could be obtained pure, provided that suitable precautions were taken in the synthesis. We had to use a large excess of concentrated methyl iodide in preparing (*6*) from its base precursor, so that quaternisation was rapid. Slow quaternisation resulted in self catalysed eliminations, giving complex mixtures from which the quaternary salt could not be isolated—a major problem throughout the project. Even with only one centre to be quaternised the likely impurities are numerous—in this case compounds (*8*), (*9*) and (*10*) are almost certain. They all arise from the elimination of compound (*6*), catalysed by the starting material and subsequent proton exchange, with quaternisation of any free base which is present.

The synthesis of analogues of (*5*) was likely to be limited by the preference for electron donating substituents such as the methoxy group in the Bischler–Napieralski cyclisation step. This was overcome quite nicely by a novel use of the Reformatski procedure in which the quaternary salt (*11*) was used in place of a ketone to give the free base of (*8*), but with the labile ester function added after the cyclisation. By using this method the bis-ester (*12*) was obtained, quaternised and sent for pharmacological evaluation.[4] To our chagrin the bis-quaternary salt was not only lacking in potency but persistent in the body. We could synthesise analogues which eliminated more quickly,[5] but this did not cure the problem. Our understanding of the chemistry was obviously incomplete, and further work showed that the elimination was reversible. At pH 7.4 the quaternary salt (*6*) and the tertiary salt (*9*) were in equilibrium.

The next step

Shortly before this stage was reached I was appointed to a lectureship in the pharmacy department at Strathclyde and John Urwin took over the MRC postdoctoral fellowship. One of our first tasks was to find a way of making the elimination irreversible and the obvious way was to allow separation of the amine and ester groups which were held in close proximity in (*7*). The reverse reaction would then become interrather than intramolecular and so would



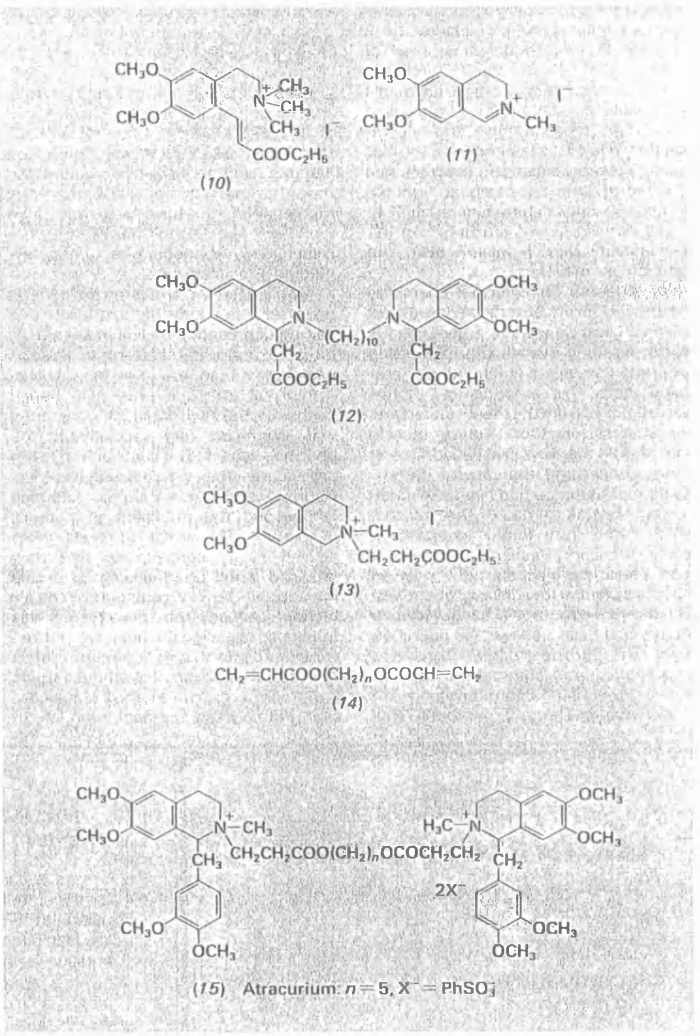
be improbable. The objective could be accomplished most readily in a structure such as (*13*), which after a certain amount of difficulty with the quaternisation was shown to eliminate in a satisfactory manner at pH 7.4. The adoption of this approach to a bis-quaternary required the synthesis of a bis-acrylate (*14*), which would add the amine 'end group' in a Michael reaction before quaternisation. The use of ethylene glycol as the centre portion of (*14*) gave a chain of 10 units which was supposed to be the optimum, as in (*4*). Compounds with this chain length and with two ester links had previously provided highly effective muscle relaxants, specifically the succinate bis-ester with choline as the quaternary moiety, known as succinyl choline or suxamethonium.

Although the bis-acrylate showed a distressing tendency to polymerise, the problem was not insuperable and (*14*) was converted to a number of bis-quaternary salts with different end groups. One of these—(*15*), which was coded BW18A by Wellcome Research Laboratories—was a very good muscle relaxant in laboratory tests.[6] It was quite potent and, above all, free from the common side effects associated with this kind of drug. Many of these desirable attributes stemmed from the use of tetrahydropapaverine as the end group, taking the structure much closer to (*4*) and to the original curare alkaloids such as (*1*), but with a substantially shorter duration of action.

At this point the MRC grant ran out and renewal was refused. Fortunately, a student approaching finals had expressed interest in doing research in isoquinoline chemistry with me and was persuaded to carry the muscle relaxant project forward. The student, George Dewar, over the next three years made a contribution to the outcome of the project which could scarcely have been surpassed.

He first showed that the Hofmann elimination rates which had been obtained were close to the limit. Faster rates could be achieved[7] but were associated with difficulties in synthesis through self-catalysed elimination. It was also probable that very fast elimination would result in most of the drug being destroyed before reaching the site of action, with a consequent loss of potency.

Up to this time, the anion X⁻ in structure (*15*) had been iodide because methyl iodide was used in quaternisation. As long as they were reasonably pure the products were pale yellow solids which were fairly easy to handle. Their solubility was limited, however, and in anticipation of a requirement for larger doses in further pharmacological tests, a request was made for a more soluble salt. After due consideration methyl methanesulphonate was used as quaternising agent, giving the more hydrophilic CH_3SO_3 anion. The product was certainly very soluble—so much so that under certain conditions it was hygroscopic, declining rapidly from a fluffy white solid to an intractable gum. This problem was solved on the laboratory scale by George running to the desiccator with the powder, freshly obtained by dripping the reaction mixture into a large volume of dry ether. Neither aspect later commended itself to the production chemists!



A considerable amount of work on analogues of BW18A showed that the only worthwhile modification was to the centre chain of (*15*), the compounds with $n = 5$ or 6 being much more potent.[8] The former (BW33A) was chosen for extensive study, at which point the chemical problems became those of scaled-up synthesis and quality control. To facilitate the former, the anion was changed once again to benzenesulphonate, avoiding the necessity for large quantities of dry ether and the resulting unacceptable fire hazard. In retrospect the iodide would probably have been perfectly acceptable, because in clinical trials the product had more than adequate potency.

Stereochemistry

The stereochemistry of BW33A was complex.[9] There were 10 possible isomers arising from four asymmetric centres, six fewer than would be expected if there were no symmetry about the middle of the chain. If the end groups were categorised as *cis* or *trans* the physically distinguishable compounds were *cis/cis, cis/trans* and *trans/trans*. These could be determined by HPLC and as long as the proportions did not vary the potency of the product was reproducible. It is probable that all the isomers contribute to the pharmacological actions of the mixture, some being more potent than others.

After a great deal of preparatory work by Wellcome Research Laboratories, BW33A became atracurium and a successful surgical muscle relaxant—Tracrium—eventually reached the market. Apart from the lack of side effects the

major significance of the drug is that its decomposition is controlled by pH. Even in patients with a defective liver or kidneys the pH of blood is closely controlled and the drug breaks down at a predictable rate. Although we have called atracurium 'biodegradable' the decomposition does not depend on any biological process except the maintenance of blood pH and the compound should perhaps be called 'chemodegradable'. In a way this represents a triumph for the predictability of chemistry over the randomness of biology.

For students of medicinal chemistry the atracurium project offers some useful lessons. Most important, a good idea is worth pursuing even if the theory and experiments which led to it are later found faulty. Three foundation stones were removed from the theory underlying the search for short acting muscle relaxants as the work proceeded. One of these, concerning the rate of decomposition of petaline, has been explained in some detail already. The second, which came from totally independent work at Wellcome Research Laboratories soon after the project started,[10] showed that the potent arrow poison constituent tubocurarine was not a bis-quaternary salt, as had been believed for nearly 40 years. The third foundation stone was the so called 'interonium distance'. As I have explained, the optimum separation of the two quaternary nitrogens was widely believed to correspond to 10 atoms, as in decamethonium, laudexium and the bis-ester succinyl choline.[1] Without a willingness to abandon this preconception and move to longer chains, atracurium would never have been made. A full assessment of the literature shows that there are sufficient exceptions to the 10 unit rule to have discredited the theory some time before atracurium was made, although those who had been working in the field for many years were reluctant to abandon one of the few guiding principles.

A second lesson concerns the general philosophy behind the synthesis and testing of potential medicinal agents. At no point up to the selection of BW33A was more than one person involved actively at any one time with bench chemistry, but each candidate compound was examined very carefully by the pharmacologists. The contribution to the project by the pharmacologists Roy Hughes and Dennis Chapple extended far beyond the provision of potency evaluation. Their test methods were arrived at by consultation and they provided a full pharmacological profile for each and every compound sent for testing: feedback from these results was then used to design the next two or three compounds. In the right kind of project the careful selection of synthetic targets must offer a greater chance of success than the shotgun approach with lots of new compounds evaluated in a superficial preliminary 'screen'.

Acknowledgements. Valuable contributions were made by Professor J. B. Stenlake, N. C. Dhar and many staff at Wellcome including K. Elcome, G. G. Coker, J. C. Lindon, A. G. Ferrige, P. H. Cobb and S. W. Poole.

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References

1. M. Martin-Smith in *Drug design*, E. J. Ariens (ed), vol 2, p 454. New York: Academic, 1971; J. B. Stenlake in *Medicinal chemistry: the role of organic chemistry in drug research*, S. M. Roberts and B. J. Price (eds), p143. New York: Academic, 1985.
2. N. J. McCorkindale *et al*, *Tetrahedron Lett.*, 1964, **51**, 3841.
3. A. Brossi *et al*, *Helv. Chim. Acta*, 1960, **43**, 583.
4. J. B. Stenlake *et al*, *Eur. J. Med. Chem.*, 1979, **14**, 77.
5. J. B. Stenlake *et al*, *Eur. J. Med. Chem.*, 1979, **14**, 85.
6. J. B. Stenlake *et al*, *Eur. J. Med. Chem.*, 1981, **16**, 508.
7. J. B. Stenlake *et al*, *Eur. J. Med. Chem.*, 1983, **18**, 273.
8. J. B. Stenlake *et al*, *Eur. J. Med. Chem.*, 1981, **16**, 515.
9. J. B. Stenlake *et al*, *Eur. J. Med. Chem.*, 1984, **19**, 441.
10. A. J. Everett, L. A. Lowe and S. Wilkinson, *J. Chem. Soc., Chem. Commun.*, 1970, 1020.

Pharmaceutical Science Communications, 4, 3–14

Chemodegradable neuromuscular blocking agents—I: *Bis*-3,4-dihydroisoquinolinium salts

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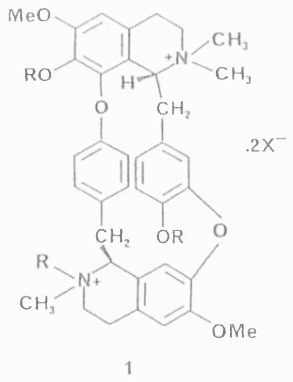
A series of *N,N'*-polymethylene-*bis*-3,4-dihydroisoquinolinium quaternary salts has been prepared and examined for neuromuscular blocking activity of short duration. Cyclodehydration of *N*-[1,3-di-(3,4-dimethoxyphenyl)prop-2-yl] formamide yielded 3,4-dihydro-3-(3,4-dimethoxybenzyl)-6,7-dimethoxyisoquinoline which, when reacted with a variety of α,ω-diiodoalkanes, gave the required agents. The synthesis of two analogues of laudexium is also described. The 3,4-dihydroisoquinolinium salts exhibited a short action in the cat, but potency was weak and accompanied by vagolytic ganglion blocking actions.

Keywords: NMB agents; *bis* quaternaries; 3,4-dihydroquinolinium salts

Introduction

D-Tubocurarine 1 (R = H; Figure 1) is the prototype anti-depolarizing neuromuscular blocking agent (NMB agent) and it still sees some clinical use, despite side-effects such as tachycardia and histamine release [1]. The corresponding *bis*-quaternary dimethyl ether ('metocurine'; 1; R = Me) is a more potent, longer acting drug but it is essentially free of the side-effects associated with the parent [2], and it is in this regard that metocurine has assumed a place of importance in structure–action studies and drug design [3]. On the other hand, suxamethonium 2 (Figure 1) displays an undesirable depolarizing mode of action but it is still used clinically because of its short duration of action in the majority of patients [4].

For many years, workers in the field have tried to identify a good, short-acting, neuromuscular



1

$$(Me)_3N^+CH_2CH_2—O—COCH_2CH_2—COOCH_2CH_2N^+(Me)_3 \cdot 2X^-$$

Figure 1. Chemical structures of tubocurarine (1; R = H), metocurine (1; R = Me) and suxamethonium.

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blocking agent (the curariform equivalent of suxamethonium); a short action in NMB agents is considered essential to avoid prolonged muscle paralysis after completion of the surgical procedure [5]. One approach has been to incorporate the tetrahydropapaverinyl feature **3** (Figure 2), seen in metocurine, into a *bis*-quaternary structure (this provides for NMB action of the anti-depolarizing type), and manipulate the inter-nitrogen chain chemically to allow for molcular fragmentation *in vivo* to yield mono-quaternary or non-quaternary species devoid of activity. A few good examples of this approach are to be found in agents **4**–**6** (Figure 2) [6–13].

This paper concentrates on the susceptibility of 3,4-dihydroisoquinolinium salts, such as **7** (R = Me, Et, Pr, etc.; Figure 3), to nucleophilic attack on position 1, with attendant loss of permanent charge [14]. From the above considerations, a target molecule would be the *bis*-1-veratryl agent **8** (Figure 3), but such a compound (e.g. $n = 10$) has been found to be of low

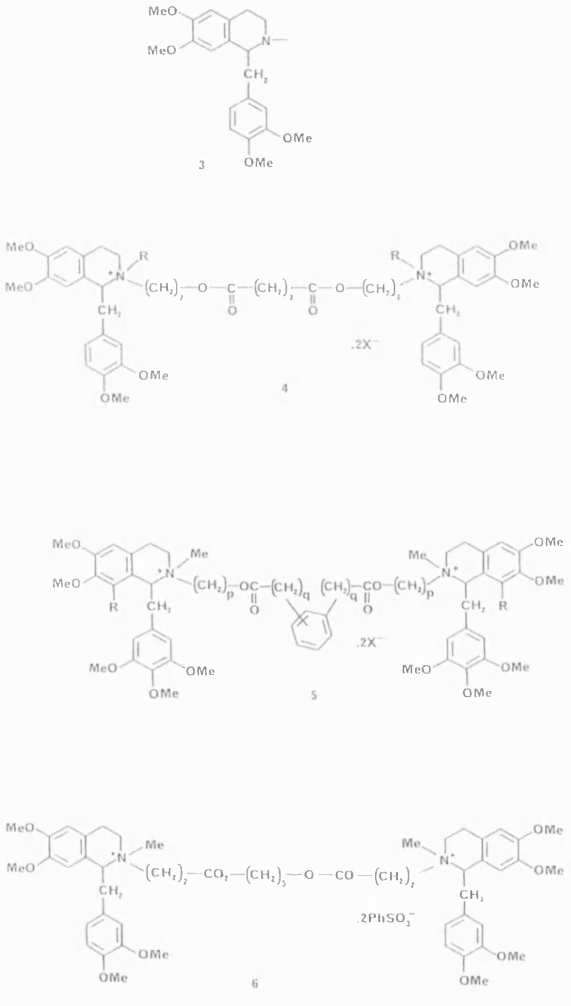


Figure 2. The tetrahydropapaverinyl unit, **3**, and some related potential short-acting, non-depolarizing neuromuscular blocking agents, **4**–**6**.

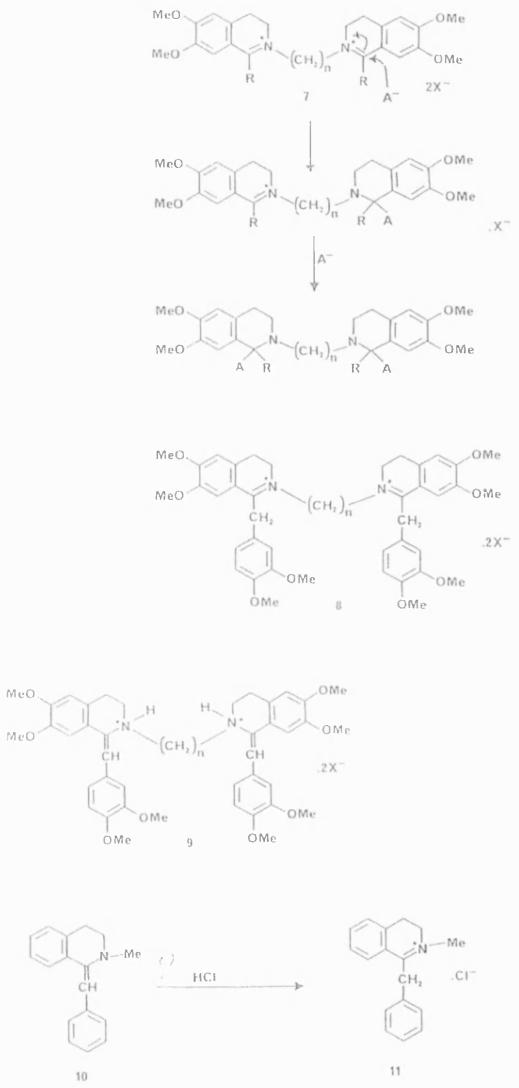
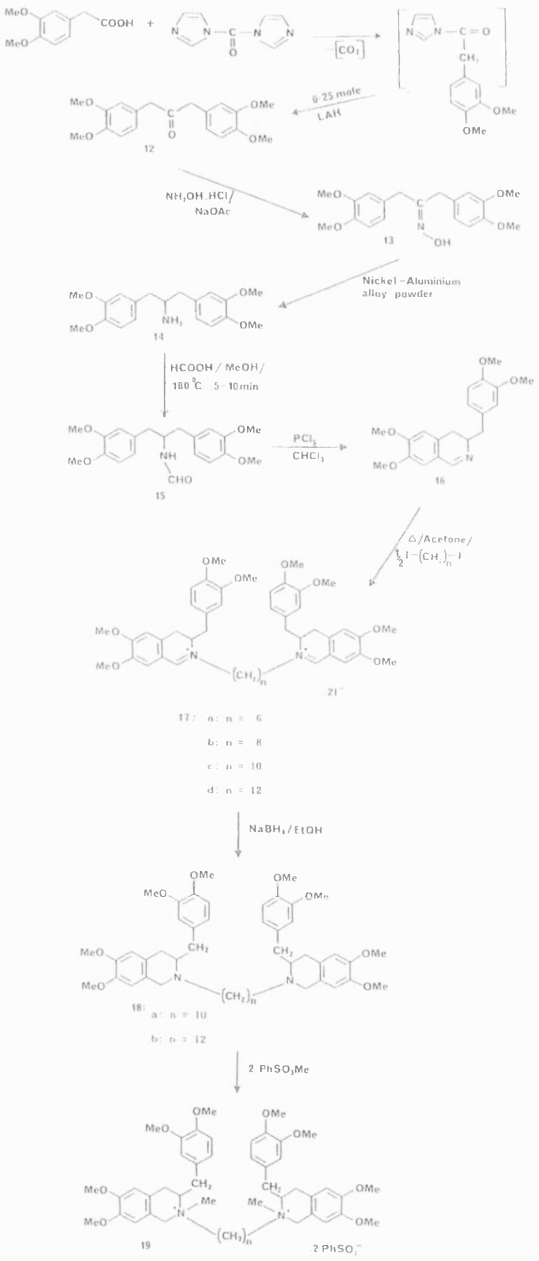


Figure 3. Some 3,4-dihydroisoquinolinium salts, together with their possible breakdown and tautomerization.

potency and severe in its side-effects [14]. This observation has been rationalized on the basis of the ability of **8** to undergo tautomerism to **9** (Figure 3), a change that loses the necessary quaternary status of the molecule. Such shift of an endocyclic double bond into conjugation between two aromatic rings is, however, well documented in 1-benzyl-3,4-dihydroisoquinolines of type **10** (Figure 3) [15–17].

We envisaged that a translocation of the veratryl unit from C-1 to C-3 of the isoquinoline ring, to give compounds **17** (Scheme 1), should circumvent the problems of tautomerism and thus give compounds with the desired short action



Scheme 1.

profile. Additionally, the tetrahydro-*bis*-quaternary compounds **19** (Scheme 1), which are isomeric to laudexium (Figure 4), were prepared to give a qualitative comparison of the change in NMB potency as a result of transposition of the veratryl function around the tetrahydroisoquinoline ring.

Materials and methods

Chemistry

Synthetic routes. The *bis*-quaternary salts **17** (see Scheme 1; includes intermediates) were prepared by treatment of 3,4-dihydro-3-(3,4-dimethoxybenzyl)-6,7-dimethoxyisoquinoline **16** with the appropriate α,ω-diiodoalkane. The intermediate diveratrylketone **12** was prepared, in the first instance, by the Corey–Seebach procedure [18, 19] (Scheme 2) which involves an *umpolung* (dipole inversion) of a carbonyl function. Thus, sequential lithiation and alkylation of 1,3-dithiane gave the 2,2-disubstituted 1,3-dithiane **21** (Scheme 2). Mercuric chloride hydrolysis [20] of the thioketal **21** afforded diveratrylketone. Repetitive column chromatography was necessary to isolate **21**, and the subsequent dethioketalization process also proved to be inefficient; the reaction pathway expressed in Scheme 2 was clearly too cumbersome for the preparation of the large quantities of diveratrylketone that were required. Elsewhere, Prudhommeaux *et al.* have previously

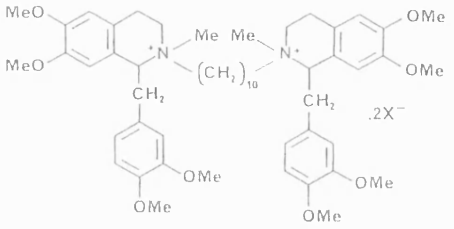
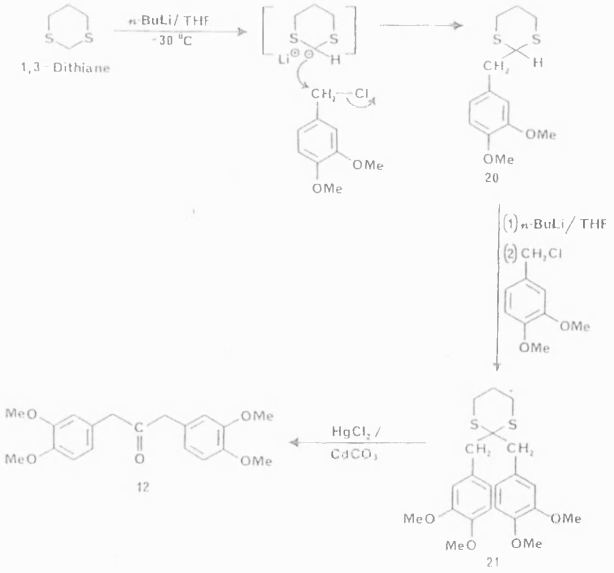


Figure 4. Chemical structure of laudexium.



Scheme 2.

reported [21] attempts to synthesize diveratrylketone by other means, and eventually achieved yields of not more than 30% by pyrolysis of the lead salt of 3,4-dimethoxyphenylacetic acid [22, 23]. In our hands this method proved messy, and yields were very low.

We therefore pursued an alternative one-step procedure (Scheme 1), previously reported by Sainsbury and Wyatt [24], to secure diveratrylketone. Addition of 0.25 mole of lithium aluminium hydride (LAH) to a decarbonated solution of 3,4-dimethoxyphenylacetic acid and *N,N'*-carbonyldiimidazole in dry THF gave diveratrylketone. Various modifications of the procedure to secure yields greater than 52% proved fruitless. Reductive amination of diveratrylketone via the intermediate oxime **13** gave diveratrylmethylamine **14**. The procedure involved *in situ* catalytic reduction of the oxime **13** with nickel–aluminium alloy powder. Formylation of diveratrylmethylamine **14**, with an equimolar quantity of formic acid gave the substituted β-phenylethylamide **15**, and standard Bischler–Napieralski cyclization [25, 26] of this with phosphorus pentachloride gave the desired 3,4-dihydroisoquinoline **16**. Treatment of **16** with half a mole-quivalent of the appropriate α,ω-diiodoalkane gave the *bis*-quaternary salts **17**. Further reduction with sodium borohyride, followed by quaternization gave the saturated *bis*-quaternary salts **19**.

Description of experiments. Melting points were recorded on a Gallenkemp melting point apparatus and are uncorrected. Infrared spectra were recorded on a Perkin–Elmer 782 (or Unicam SP1025) instrument and were obtained on liquid films or as KCl discs (for solids). ^{1}H magnetic resonance spectra were recorded on JEOL JNM 270 FT and JEOL GX 400 FT instruments. ^{13}C magnetic resonance spectra were recorded on a JEOL JNM 270 FT instrument operating at 67.8 MHz. $CDCl_3$ was the standard solvent of choice (unless indicated otherwise), with TMS as internal standard. Mass spectra were recorded on a VG analytical Micromass 7070E spectrophotometer (70 eV EI, CI and FAB). Where appropriate, column chromatography was performed using Merck silica gel (Kieselgel 60: 230–400 mesh). Unless stated otherwise, all reagents are commercially available. IR and NMR data for the *bis*-3,4-dihydroisoquinolinium salts were recorded and are available on request from the authors (GHD and SSP).

3,4-Dimethoxybenzylchloride (veratryl chloride). Concentrated etheral hydrochloric acid solution (32 ml) was added dropwise at 0°C to a solution of 3,4-dimethoxybenzylalcohol (20 g, 119 mmol; secured by standard sodium borohydride reduction of veratraldehyde) in dry diethyl ether (30 ml). The mixture was left standing at 1–2°C for 24 h, after which it was concentrated *in vacuo* to an oil. Crystallization from light petroleum (bp 30–40) gave colourless plate crystals of the title compound (17.9 g, 80.7%).

Mp: 49–50°C ([27] 50–50.5°C).
IR ν_{max}(KBr) cm^{-1}: 3020, 2950, 2840, 1615, 1610, 1520.
s_H: 6.90 (3H, *m*, Ar-H); 4.58 (2H, *s*, -CH_2-); 3.90 (6H, *s*, 2 × OMe).

2,2-Di-(3,4-dimethoxybenzyl)-1,3-dithiane, **21**. Prepared by the Corey–Seebach procedure [18]: *n*-butyllithium (9.35 ml of a 2.6 M solution in hexane; 25 mmol) was added dropwise to a stirred solution of 1,3-dithiane (2.6 g, 22 mmol) in dry THF (100 ml) under nitrogen at −30°C. After 2 h, veratryl chloride (4.2 g, 22.5 mmol) in dry THF (60 ml) was added dropwise over 5 min at −1°C. The yellow solution was stirred at 8°C for 70 h after which *n*-butyllithium (9.35 ml of a 2.6 M solution in hexane, 25 mmol) was added dropwise at −30°C, and stirred at −30°C for a further 3 h. To the resultant red/maroon solution was added, dropwise, veratryl chloride (4.2 g, 22.5 mmol) in dry THF (60 ml) over 5 min at −10°C. The solution regained its yellow colour and was stirred for a further 16 h at 0°C, poured into three times its volume of water, acidified to pH 5–6 and extracted with diethyl ether (5 × 200 ml). The combined extracts were washed with 3% sodium bisulphite (2 × 200 ml), 5% potassium hydroxide (2 × 100 ml), water and dried ($MgSO_4$). Evaporating the solvent *in vacuo* gave a dark yellow oil. Column chromatographic separation (diethyl ether: light petroleum, bp 60–80; 1:3) and subsequent crystallization from ethanol gave colourless plate crystals of the thioketal (3.5 g, 37.9%).

Mp: 101.5–102°C.
IR ν_{max}(KBr) cm^{-1}: 2980, 2860, 1610, 1530.
s_H: 6.90–6.80 (6H, *m*, Ar-H); 3.90–3.80 (12H, *s*, 4 × OMe); 3.15 (4H, *s*, 2 × Ar-CH_2-); 2.80 (4H, *t*, 2 × -CH_2-S); 1.90 (2H, *m*, -CH_2-).
MS (CI): *m/z* 421 (86%, $M^+ + 1$).

The column chromatographic procedure also yielded the corresponding mono-substituted dithiane, and the following data were secured for 2-(3,4-dimethoxybenzyl)-1,3-dithiane, **20**:

IR ν_{max}(KBr) cm^{-1}: 2900, 2870, 1620, 1605, 1535.
s_H: 6.80 (3H, *m*, Ar-H); 4.24 (1H, *t*, J = 7.4 Hz, -CH-); 3.84 (6H, *m*, 2 × OMe); 3.00 (2H, *d*, J = 7.4 Hz, Ar-CH_2); 2.90–2.80 (4H, *m*, 2 × -CH_2-S); 2.20–2.00 (2H, *m*, S-CH_2-CH_2-).
MS (CI): *m*/z 271 (90%, $M^+ + 1$).

1,3-Di-(3,4-dimethoxyphenyl)propan-2-one (diveratrylketone), **12**.

(a) By hydrolysis of **21** [20]
A mixture of 2,2-di-(dimethoxybenzyl)-1,3-dithiane **21** (0.5 g, 1.2 mmol), mercuric chloride (0.68 g, 2.5 mmol) and cadmium carbonate (0.4 g, 2.3 mmol), in methanol (30 ml), was heated at 55°C for 24 h. After cooling, the mixture was filtered, and the filtrate extracted with chloroform (2 × 100 ml). The combined organic extracts were washed (water), dried ($MgSO_4$) and the solvent removed *in vacuo* to leave a yellow oil which solidified on standing overnight. Crystallization from 95% ethanol gave yellow star crystals of diveratrylketone (0.23 g, 58%).

(b) Sainsbury and Wyatt procedure [24]
To a stirred solution of *N,N'*-carbonyldiimidazole (16.5 g, 102 mmol) in dry THF (200 ml) was added 3,4-dimethoxyphenylacetic acid (20 g, 102 mmol). Carbon dioxide was evolved and when this had subsided, dry nitrogen was bubbled through the mixture for 30 min. The resulting yellow solution was cooled to −10°C and lithium aluminium hydride (1 g, 26 mmol) added in small portions. The reaction mixture was allowed to warm to room temperature and stirred for 8 h. A further quantity of THF (150 ml) was added, followed by water until all bubbling had completely subsided. The mixture was filtered and washed through with copious quantities of chloroform. The aqueous layer from the filtrate was isolated and extracted with chloroform (3 × 200 ml). The extracts were combined with the organic layer, washed (water), dried ($MgSO_4$) and the solvent evaporated *in vacuo* to give the ketone **12** as an oil, which slowly crystallized on standing (8.45 g, 51%).

Mp: 98–99°C (methanol) ([28] 98–99°C).
IR ν_{max}(KBr) cm^{-1}: 3040, 2980, 2860, 1730, 1600, 1540. s_H: 6.80 (2H, *d*, J_{ortho} = 8 Hz, (2H, *dd*, $J_{ortho,meta}$ = 8 Hz and 1.9 z, 2 × 6-Ar-H); 6.60 (2H, *d*, J_{meta} = 1.9 Hz, 2 × 2-Ar-H); 3.82 (12H, *s*, 4 × OMe); 3.64 (4H, *s*, 2 × -CH_2-).
MS (70 eV EI): *m*/z 330 (20%, M^+).

2-Amino-1,3-di-(3,4-dimethoxyphenyl)propane (diveratrylmethylamine), **14**. Diveratrylketone (5.1 g, 15.5 mmol) was heated under reflux in methanol (50 ml) with hydroxylamine hydrochloride (1.56 g, 22.4 mmol) and sodium acetate (3.27 g, 39.9 mmol) for 6 h. After cooling, the mixture was made alkaline to litmus paper with 5 N sodium hydroxide solution, and nickel–aluminium alloy powder (6.56 g, 76 mmol) was added cautiously over 0.5 h. The mixture was then stirred for 4 h, filtered, and washed through with copious quantities of chloroform (4 × 100 ml). From the filtrate, the aqueous layer was separated and extracted with chloroform (4 × 50 ml). The extracts were combined with the organic layer, washed (water), dried ($MgSO_4$), and the solvent evaporated *in vacuo* to give a yellow oil. Crystallization from light petroleum (bp 60–80) gave the title compound as colourless flakes (4.3 g, 87%).

Mp: 83–84°C ([28] 88–89°C).
IR ν_{max}(KBr) cm^{-1}: 2950, 2840, 1600, 1530.
s_H: 6.80 (6H, *m*, Ar-H); 3.86 (12H, *s*, 4 × OMe); 3.20 (1H, *m*, CH); 2.80 (2H, *dd*, J = 11.25 Hz and 3.38 Hz -CH_2-); 2.50 (2H, *dd*, J = 11.25 Hz and 7.31 Hz -CH_2-); 1.40–1.20 (2H, *s*, NH_2: exchangeable with D_2O).
MS (CI): *m*/z 332 (100%, $M^+ + 1$).

N-[1,3-di-(3,4-dimethoxyphenyl)prop-2-yl]formamide, **15**. Formic acid (0.5 g, 10.9 mmol) was added to a solution of the amine **14** (4.2 g, 12.7 mmol) in methanol (10 ml) and left to stand for 15 min. Evaporation of the solvent *in vacuo* gave a yellow solid which was heated at 180°C for 10 min to give a dark brown oil. Crystallization from 95% ethanol gave fine, colourless crystals of the title compound (3.9 g, 85.5%).

Mp: 124°C ([21] 129–130°C).
IR ν_{max}(KBr) cm^{-1}: 3300, 2960, 2850, 1680, 1620, 1600, 1530.
s_H: 8.06 (1H, *s*, -CHO); 6.85–6.60 (6H, *m*, Ar-H); 5.45 (1H, *d*, -HN-); 4.55–4.45 (1H, *m*, -CH-); 3.85 (12H, *s*, 4 × OMe); 2.90–2.60 (4H, *m*, 2 × -CH_2-).
MS (70 eV EI): *m*/z 359 (22%, M^+).

3,4-Dihydro-3-(3,4-dimethoxybenzyl)-6,7-dimethoxyisoquinoline, **16**. Phosphorus pentachloride (3 g, 14.4 mmol) was added to a stirred solution of the amide **15** (2 g, 5.6 mmol) in dry chloroform (10 ml) and stirred at room temperature for 24 h. Dry diethyl ether (60 ml) was then added to give a flocculent precipitate. This was filtered off and recrystallized from propan-2-ol to give a yellow crystalline proton salt of the title compound (1.8 g, 85%). A small quantity of the proton salt was liberated to the free base and converted to the corresponding hydrobromide salt.

Mp (HBr salt): 270–271°C ([21] 272–273°C).
IR (HBr salt)ν_{max}(KBr) cm^{-1}: 2960, 2860, 1640, 1615, 1530.
δ_H (free base): 8.25 (1H, *d*, 1-Ar-H); 6.85–6.75 (4H, *m*, 2'-, 5'-, 6'- and 8-Ar-H); 6.60 (1H, *s*, 5-Ar-H); 3.90–3.75 (13H, *m*, 4 × OMe and 3-H); 3.20 (1H, *dd*, 9a-H, J = 13.50 Hz-with 9b-H, J = 5.63 Hz-with 3-H); 2.75 (1H, *dd*, 9b-H, J = 13.50 Hz-with 9a-H, J = 9.00 Hz-with 3-H); 2.60 (1H, *dd*, 4_{eq}-H, J = 15.75 Hz-with 4_{ax}-H, and J = 5.63 Hz-with 3-H); 2.45 (1H, *dd*, 4_{ax}-H, J = 15.75 Hz-with 4_{eq}-H and J = 11.25 Hz-with 3-H).
MS (70 eV EI): *m/z* 341 (10%, M^+).

α,ω-Bis-[3,4-dihydro-3-(3,4-dimethoxybenzyl)-6,7-dimethoxyisoquinolinium]alkanes, **17**. The general method adopted was a modification of that employed by Stenlake *et al*. [14].

Example: *1,10-bis*-[3,4-dihydro-3-(3,4-dimethoxybenzyl)-6,7-dimethoxyisoquinolinium] decane diiodide, **17c**. A mixture of 3,4-dihydro-3-(3,4-dimethoxybenzyl)-6,7-dimethoxyisoquinoline **16** (2.07 g, 6 mmol) and 1,10-diiododecane (790 mg, 2 mmol) in acetone (15 ml) was heated under reflux in a nitrogen atmosphere for 48 h. After cooling, the *bis*-quaternary salt was isolated by allowing the solution to drip through a sinter funnel into rapidly stirred pre-filtered dry diethyl ether. The resulting flocculent solid was filtered off, washed well with dry diethyl ether, and dried in a vacuum dessicator for 24 h in the presence of phosphorus pentoxide to give 1,10-*bis*-[3,4-dihydro-3-(3,4-dimethoxybenzyl)-6,7-dimethoxyisoquinolinium]decane diiodide, **17c**, as a yellow powder.

Yield: 96%; Mp 100–102°C Dec.
$C_{50}H_{66}I_2N_2O_8$: C,H,N.

Data for other members of the series (as above):

17a: 95%; 134°C Dec.
$C_{46}H_{58}I_2N_2O_8$: C,H,N.
17b: 98%; 94–96°C Dec.
$C_{48}H_{62}I_2N_2O_8$: C,H,N.
17d: 97%; 98–100°C Dec.
$C_{52}H_{70}I_2N_2O_8$: C,H,N.

α,ω-Bis-[1,2,3,4-tetrahydro-3-(3,4-dimethoxybenzyl)-6,7-dimethoxyisoquinolin-2-yl]alkane methyl benzenesulphonates, **19**.

Example: 1,10-*bis*-[1,2,3,4-tetrahydro-3-(3,4-dimethoxybenzyl)-6,7-dimethoxyisoquinolin-2-yl] decane dimethyl benzenesulphonate, **19a**. To a solution of the *bis*-3,4-dihydroisoquinolinium salt **17c** (4 g, 3.7 mmol) in 95% ethanol (50 ml) was added sodium borohydride (10 g, 0.2 mmol) in small portions over 0.5 h. The mixture was stirred for 24 h, quenched with dilute hydrochloric acid (10%), subsequently made alkaline to litmus paper with 5 N sodium hydroxide solution, and extracted with diethyl ether (3 × 100 ml). The combined organic extracts were washed (water), dried ($MgSO_4$), and evaporated *in vacuo* to give the corresponding reduced compound as a crude yellow oil. This was converted to the oxalate salt, and the resultant flocculent precipitate filtered off and recrystallized to give the tertiary proton salt **18a**. The free base (liberated by dissolving the dioxalate salt in water, basifying with ammonia and extracting with diethyl ether) (480 mg, 0.6 mmol) was treated with methyl benzenesulphonate (0.3 g, 1.7 mmol) at room temperature for 24 h to give 1,10-*bis*-[1,2,3,4-tetrahydro-3-(3,4-dimethoxybenzyl)-6,7-dimethoxyisoquinolin-2-yl]decane dimethyl benzenesulphonate **19a**, which was isolated as a colourless powder by precipitation from dry diethyl ether according to the same procedure used for the *bis*-3,4-dihydroisoquinolinium salts above.

Yield: 96%; Mp 98–104°C Dec.
$C_{64}H_{84}N_2O_{14}S_2 . 2H_2O$: C,H,N.
19b: 98%; 113–116°C Dec.
$C_{66}H_{88}N_2O_{14}S_2 . 4H_2O$: C,H,N.

Pharmacology

Neuromuscular blocking potencies, vagal and ganglion blocking actions, and other cardiovascular data were determined in an anaesthetized cat according to the procedure described

elsewhere [29]. The results are presented in Table 1. All pharmacological data were obtained on the racemic mixtures secured from chemical work-up procedures.

Pharmacological results

By translocating the veratryl moeity from the C-1 to C-3 position of the isoquinoline ring, we had anticipated two advantages: firstly, the favourable potency and hydrophilic–lipophilic balance associated with laudexium (Figure 4) is potentially maintained in the new compounds, and, secondly, previous work [14,30] on 3,4-dihydroisoquinolinium compounds has shown that 3-alkyl substitution in itself enhances potency, and therefore we expected a further increase in potency of the new compounds.

Three of the 3,4-dihydroisoquinolinium compounds, **17a–c**, exhibited neuromuscular blocking action of short duration (*ca* 10 min; Figure 5). The minimum dose required was 1.6 mg/kg (Table 1), thus suggesting a much reduced potency in these compounds compared to similar 3,4-dihydroisoquinolinium salts reported by other workers [14, 31]. Furthermore, at paralysing doses, **17b** markedly lowered blood pressure, and atropine-like effects were exhibited by all three active compounds. **17b** and **17c** also showed ganglion blocking action; surprisingly, this action is virtually absent in **17a** (*bis*-onium compounds with a six-atom inter-onium spacing are usually associated with peak ganglion blocking action [31]). The dodecamethylene analogue **17d** lacked neuromuscular blocking action at doses up to 1.6 mg/kg, but produced severe cardiovascular disturbances at these doses.

Conclusion

In comparison to other *bis*-3,4-dihydroisoquinolinium salts **7** and **22** (Figure 6) [30], the markedly reduced potencies observed in our compounds may be explained as follows: firstly, nucleophilic attack at C-1 in compounds **7** and **22** may be hindered by substituents present at C-1 whereas in **17** access to nucleophiles remains unhindered, and this may allow for a more rapid attack. Consequently, the low neuromuscular blocking potency of compounds **17** may well be an expression of rapid *in vivo* breakdown, so that the proportion of intact molecules actually reaching the site of action is low in relation to the injected dose.

Secondly, it is quite likely that the conformation adopted by the veratryl moiety imposes a steric hindrance to the interaction of the onium head with the receptor site. This is evident from an approximate comparison of the neuromuscular blocking potency of the *bis*-quaternary salt **19a** with that of laudexium (Figure 4), which

Table 1. Neuromuscular blocking potencies and cardiovascular effects of *N,N'*-polymethylene-*bis*-3,4-dihydroisoquinolinium (**17**) and *N,N'*-polymethylene-*bis*-1,2,3,4-tetrahydroisoquinolinium (**19**) quaternary salts in the cat[a]

Compound	Dose[b]	% Neuromuscular block		% Vagal block	% Ganglionic block[c]	% Change[d] in	
		Soleus	Tibialis			BP	HR
17a	1600	26	52	24	6	6	5
17b	1600	70	89	30	16	35	12
17c	1600	21	60	44	19	**	**
17d	1600	*	*	27	*	25	38
19a	200[e]	92	75	0	0	0	2
19b	400[e]	93	100	37	7	15	3

[a]Individual results ($n = 1$).
[b]Cumulative dose in μg/kg.
[c]Ganglionic blockade indicated by contractility changes of nictitating membrane.
[d]Changes in blood pressure (BP) and heart rate (HR); bold numbers indicate an increase in % change.
[e]Bolus dose in μg/kg.
*No effect on muscles, vagus or nictitating membrane.
**No effect on the blood pressure or heart rate.

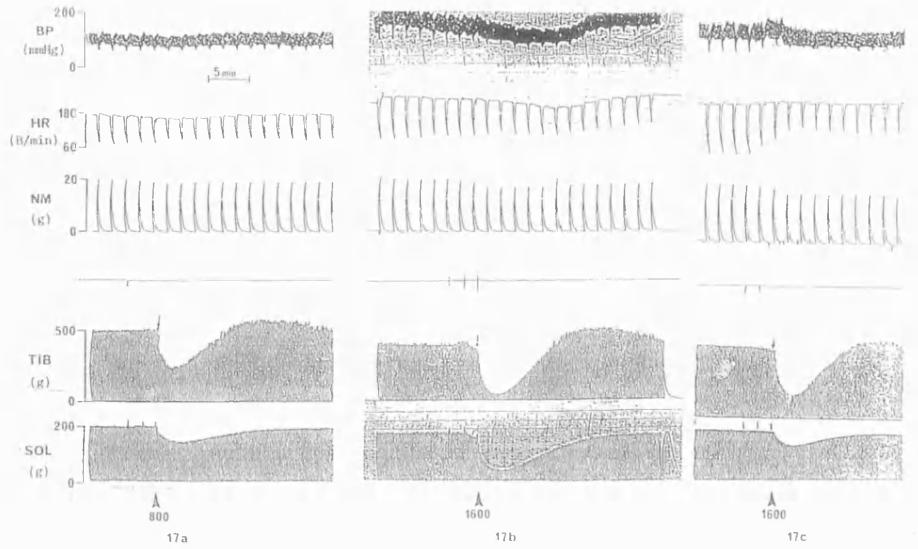
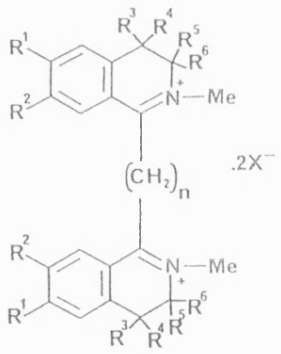


Figure 5. Effects in a cat of *bis*-3,4-dihydroisoquinolinium salts (**17**) on arterial blood pressure (BP), heart rate (HR), contractions (upward deflections) of the nictitating membrane (NM) to preganglionic stimulation (2–5 Hz for 10 s every 100 s), and maximal contractions (upward deflections) of the tibialis anterior (TIB) and soleus (SOL) muscles evoked by stimulation of motor nerves (0.1 Hz). The right vagus nerve was stimulated every 100 s for 10 s at 5–10 Hz to produce decrease in heart rate (downward deflection of the HR trace). Neuromuscular blocking profiles shown are at doses of μg/kg.



22

Figure 6. Structure of some 1,1′-linked *bis*-3,4-dihydroisoquinolinium salts, **22** [30].

indicates that the latter is approximately 1.5 times more potent than its 3-veratryl isomer. This difference in potency as a result of the translocation of the veratryl moiety to the C-3 is highlighted further in the second paper of this series.

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References

1. Maclagen J. Competitive neuromuscular blocking drugs. In: Zaimis E, ed. *Neuromuscular Junction. Handb. Exp. Pharm.*, Vol. 42, pp. 421–486. Berlin: Springer-Verlag, 1976.

2. Hughes R and Chapple DJ, 1976, Effect of nondepolarizing neuromuscular small blocking agents on peripheral autonomic mechanisms in cats. *British Journal of Anaesthesiology*, **48**, 59–68.
3. Taylor EP, 1952, Synthetic neuromuscular blocking agents. Part II. *Bis*-(quaternary ammonium salts) derived from laudanosine. *Journal of the Chemical Society*, 142–145.
4. Durant NN and Katz RL, 1982, Suxamethonium. *British Journal of Anaesthesiology*, **54**, 195–208.
5. Savarese JJ and Kitz RJ, 1975, Does clinical anaesthesia need new neuromuscular blocking agents? *Anaesthesiology*, **42**, 236–239.
6. Gladych JMZ and Taylor EP, 1962, Synthetic neuromuscular blocking agents. Part IV. Compounds related to both laudexium and suxamethonium. *Journal of the Chemical Society*, 1481–1487.
7. Savarese JJ, Kitz RJ and Ginsburg S, 1980, *Bis*(isoquinolinium) compounds, compositions and methods of use. U.S. Patent No. 4 235 906. [*Chem. Abs.*, 1981, **95**, 98118d], 14 pp.
8. Savarese JJ, Kitz RJ and Ginsburg S, 1980, Neuromuscular blocking agents. U.S. Patent No. 4 192 877. [*Chem. Abs.*, 1980, **93**, 132674c], 20 pp.
9. Savarese JJ and Kitz RJ, 1973, The quest for a short-acting non-depolarizing neuromuscular blocking agent. *Acta Anaesth. Scanda. Suppl.*, **53**, 43–58.
10. Savarese JJ and Wastila WB, 1979, Pharmacology of BW985U: a short-acting nondepolarizing neuromuscular blocking agent. *Anaesthesiology*, **51**, S277.
11. Ali HH, Savarese JJ, Basta SJ, Sunder N and Gionfriddo M, 1983, Evaluation of cumulative properties of three new non-depolarizing neuromuscular blocking drugs: BWA444U, atracurium and vecuronium. *British Journal of Anaesthesiology*, **55**, 107S–111S.
12. Hughes R and Payne JP. Atracurium. In: Kharkevich DA, ed. *New Neuromuscular Blocking Agents. Handb. Exp. Pharm.*, Vol. 79, pp. 637–650. Berlin: Springer-Verlag, 1986.
13. Stenlake JB. Biodegradation and elimination of neuromuscular blocking agents. In: Kharkevich DA, ed. *New Neuromuscular Blocking Agents. Handb. Exp. Pharm.*, Vol. 79, pp. 263–276. Berlin: Springer-Verlag, 1986.
14. Stenlake JB, Waigh RD, Urwin J, Dewar GH, Hughes R and Chapple DJ, 1981, *Bis*(3,4-dihydroisoquinolinium) salts as potential neuromuscular blocking agents. *European Journal of Medical Chemistry*, **16**, 503–507.
15. Hamilton EEP and Robinson R, 1916, LXXII—An extension of the theory of addition to conjugated unsaturated systems. Part I. Note on the constitution of the salts of 1-benzylidene-2-methyl-1:2:3:4-tetrahydroisoquinoline. *Journal of the Chemical Society*, **109**, 1029–1038.
16. Koepfli JB and Perkin WH, 1928, Synthesis of oxydehydrocorydaline. *Journal of the Chemical Society*, 2989–3000.
17. Bills JL and Noller CR, 1948, Some dihydroisoquinolines and their absorption spectra. *Journal of the American Chemical Society*, **70**, 957–962.
18. Corey EJ and Seebach D, 1965, Synthesis of 1-*n*-dicarbonyl derivatives using carbanions from 1,3-dithianes. *Angew. Chem. Int. Ed. Engl.*, **4**, 1077–1078.
19. Seebach D and Kolb M, 1974, Umpolung (dipole inversion) of carbonyl reactivity. *Chem. Ind.*, 687–692.
20. Corey EJ and Crouse D, 1968, Application of *Bis*thio carbanions to the elaboration of 2-cyclohexenone systems. *Journal of Organic Chemistry*, **33**, 298–300.
21. Prudhommeaux E, Ernouf G, Foussard-Blanpin O and Viel C, 1975, Isoquinolines. 12. New structural analogs of papaverine. 8. 3-Benzyl-6,7-dimethoxyisoquinolines (di- and tetrahydro). *European Journal of Medical Chemistry*, **10**, 19–28.
22. Sugasawa S, Kakemi K and Kazumi H, 1940, Studien zur synthese von dibenzochinolizin-derivaten (III). Synthese von 3′.4′.3″.4″-tetramethoxy-1.4.5.8-tetrahydro-[1′.6′:2.3; 1″.6″:6,7-dibenzochinolizin. *Chem. Ber.*, **73**, 782–789.
23. Kenner J and Morton F, 1939, Conversion of carboxylic acids into ketones with the aid of their lead salts. *Ber.*, **72**, 452–456.
24. Sainsbury MM and Wyatt J, 1979, Electrochemical oxidation of aromatic ethers. Part 5. Further studies of the coupling reactions of alkoxylated aralkyl and aryl amides. *Journal of the Chemical Society, Perkin Trans.* **I**, 108–114.
25. Whaley WM and Govindachari TR. The preparation of 3,4-dihydroisoquinolines and related compounds by the Bischler–Napieralski reaction. In: Adams R, Adkins H, Blatt AH, Cope AC, McGrew FC, Niemann C and Snyder HR, eds. *Organic Reactions*, Vol. 6, pp. 74–150. New York: John Wiley, 1951.
26. Bischler A and Napieralski B, 1893, Zur kenntniss einer neuen isochinolin-synthese. *Ber.*, **26**, 1903–1908.
27. Baddeley G and Smith NHP, 1961, Interdependence of molecular conformation and conjugation in aromatic ethers. Part IV. *Journal of the Chemical Society*, 2516–2519.
28. Kakemi K, 1940, III. Synthesis of 3′,4′,3″,4″-tetramethoxy-1,4,5,8-tetrahydro-1′,6′,2,3,1″,6″,6,7-dibenzoquinolizine. *Journal of the Pharmaceutical Society of Japan*, **60**, 11–21.
29. Muir AW, Houston J, Marshall RJ, Bowman WC and Marshall IG, 1989, A comparison of the neuromuscular blocking and automic effects of two new short-acting muscle relaxants with those of succinlycholine in the anaesthetized cat and pig. *Anaesthesiology*, **70**, 533–540.
30. Copp FC, Coker GG, Green AF, Hughes R and Nimmo-Smith RH, 1972, Two new short-acting non-depolarizing neuromuscular blocking agents *Experimentia*, **28**, 47–48.
31. Paton WDM and Zaimis EJ, 1951, Paralysis of

autonomic ganglia by methonium salts. *British Journal of Pharmacology and Chemotherapy*, **6**, 155–168.

Microanalyses

2,2-Di-(3,4-dimethoxybenzyl)-1,3-dithiane, **21**.
Found: C, 63.0; H, 6.73%;
$C_{22}H_{28}O_4S_2$ requires C, 62.8; H, 6.71%.

2-Amino-1,3-di-(3,4-dimethoxyphenyl)propane (diveratrylmethylamine), **14**.
(Free base):
Found: C, 68.6; H, 7.32; N, 3.71%;
$C_{19}H_{25}NO_4$ requires C, 68.9; H, 7.60; N, 4.23%.

N-[1,3-di-(3,4-dimethoxyphenyl)prop-2-yl]formamide, **15**.
Found: C, 66.5; H, 6.91; N, 3.71%,
$C_{20}H_{25}NO_5$ requires C, 66.8; H, 7.01; N, 3.90%.

α,ω-Bis-[3,4-dihydro-3-(3,4-dimethoxybenzyl)-6,7-dimethoxyisoquinolinium]alkane diiodides, **17**.

17a
Found: C, 52,4; H, 5.58; N, 2.67%;
$C_{46}H_{58}I_2O_8 \cdot 2H_2O$ requires C, 52.3; H, 5.91; N, 2.65%.

17b
Found: C, 52.8; H, 5.88; N, 2.35%;
$C_{48}H_{62}I_2O_8 \cdot 2H_2O$ requires C, 53.1; H, 6.13; N, 2.58%.

17c
Found: C, 54.5; H, 5.93; N, 2.40%;
$C_{50}H_{66}I_2O_8 \cdot H_2O$ requires C, 54.8; H, 6.26; N, 2.56%.

17d
Found: C, 54.8; H, 6.26; N, 2.23%;
$C_{52}H_{70}I_2N_2O_8 \cdot 2H_2O$ requires C, 54.7; H, 6.54; N, 2.46%.

α,ω-Bis-(1,2,3,4-tetrahydro-3-(3,4-dimethoxybenzyl)-6,7-dimethoxyisoquinolin-2-yl]alkane methyl benzensulphonates, **19**.

19a
Found: C, 63.4; H, 7.02; N, 1.64%;
$C_{64}H_{84}N_2O_{14}S_2 \cdot 2H_2O$ requires C, 63.8; H, 7.36; N, 2.32%.

19b
Found: C, 62.4; H, 7.06; N, 2.27%;
$C_{66}H_{88}N_2O_{14}S_2 \cdot 4H_2O$ requires C, 62.4; H, 7.62; N, 2.21%.

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Actions of Two Dopamine Derivatives at Adreno- and Cholinoceptors

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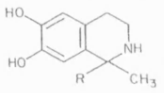
Salsolinol (1) and 6,7-dihydroxy-1,1-dimethyl-1,2,3,4-tetrahydroisoquinoline hydrobromide (2) were synthesized and their effects at adreno- and cholinoceptors investigated both in vivo and in vitro. Both 1 and 2 produced agonist effects at cholinoceptors and α- and β-adrenoceptors. Neuromuscular blocking actions were evident in vitro. Compound 2 exhibited anticholinesterase properties both in vivo and in vitro. These results indicate that dopamine derivatives of this type exhibit not only sympathomimetic activity but also complex actions at cholinoceptors.

The pharmacological activity of certain tetrahydroisoquinolines has long been established.[1] Within the series of 1-substituted 6,7-dihydroxytetrahydroisoquinolines there are several active sympathomimetic amines,[2,3] one of which, trimetoquinol, is a potent bronchodilator.[4-7] To date, all the reported findings with compounds of this series have concerned their activity at adrenoceptors. We have found that, in addition to actions at adrenoceptors, several of these compounds exhibit activity at cholinoceptors. This paper describes the effects of 1-methyl- (1)



1, R = H
2, R = CH_3

and 1,1-dimethyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (2).

Chemistry. Salsolinol (1) hydrobromide was synthesized by the method of Craig et al.[2] with minor modifications. 6,7-Dihydroxy-1,1-dimethyl-1,2,3,4-tetrahydroisoquinoline (2) hydrobromide was prepared from one of the intermediates in the synthesis of salsolinol (1). Thus, 3,4-dihydro-6,7-dimethoxy-1-methylisoquinoline was quaternized with benzyl bromide, the quaternary salt was treated with an excess of methylmagnesium iodide, and the 1,1-dimethyltetrahydroisoquinoline was hydrogenated over 10% palladium on charcoal to effect N-debenzylation. Demethylation with refluxing, constant-boiling hydrobromic acid gave the required product. The hydrochloride salt, prepared by a different route, was used by Gray et al.[8]

Results

(A) In Vivo. In anesthetized cats, both 1 and 2 (0.2–3.0 mg/kg) produced dose-related falls in mean blood pressure, but only 1 caused a fall in heart rate. These effects were antagonized by atropine (1 mg/kg).

In atropinized animals, both 1 and 2 caused dose-related elevations in mean blood pressure that were blocked by phentolamine (2 mg/kg). Compound 1 produced a reduction in the tension and degree of fusion of the incomplete tetanic contractions of the soleus muscle, an effect antagonized by propranolol (0.4 mg/kg). In contrast, 2 induced fasciculations in and augmented (by up to 100% over control) twitches of the skeletal muscle.

(B) In Vitro. In the chick biventer cervicis preparation, 1 (10–200 μg/mL) produced initial twitch augmentation, followed by blockade accompanied by a slowly developing contracture. Responses to exogenous carbachol were unaffected while those to acetylcholine were augmented. The neuromuscular blockade was unable to be reversed by choline, caffeine, physostigmine, or tetanus. Compound 2 (1–10 μg/mL) produced a similar twitch augmentation accompanied by slight contracture. There was, however, no twitch blockade. Concentrations of 2 in excess of 10 μg/mL induced contractions that were rapid in onset, readily maintained, easily reversed by washing, but poorly reproducible.

Table I. Rates of Hydrolysis of Acetylthiocholine by Homogenates of Chick Biventer Cervicis Muscle at 37 °C in the Presence and Absence of 1 and 2[a]

	concn		rate of hydrolysis (μmol min^{-1} g^{-1})	
drug	μg/mL	molar	control	drug treated
1	333	1.3×10^{-3}	6.82 ± 0.15	7.48 ± 0.14
2	10	3.7×10^{-5}	7.01 ± 0.12	3.55 ± 0.09

[a] Values quoted are the mean ± SE of $n = 4$.

In the guinea pig ileum, 1 rarely produced contraction whereas 2 consistently did so. The resulting log concentration–effect curves for 2 were parallel to those produced by carbachol with similar maxima (Figure 1). The contractions elicited by 2 and carbachol were readily reversed by washing. Atropine antagonized equally the effects of both agonists (Figure 1). Compound 2 was approximately 85 times less potent than carbachol when calculated at the $E_{max} = 50$ level. Concentrations of hexamethonium that antagonized the action of nicotine were without effect on 2.

In chick biventer cervicis homogenates, 1 in concentrations up to 333 μg/mL (1.3×10^{-3} M; the highest practical concentration) caused no inhibition of the rate of hydrolysis of acetylthiocholine by esterase enzymes (Table I). Compound 2 produced 50% inhibition (I_{50}) of hydrolysis at 10 μg/mL (3.7×10^{-5} M) (Table I). Thus, in comparison with physostigmine that has a reported I_{50} of 2×10^{-8} M,[9] compound 2 was approximately 540 times less potent as an anticholinesterase.

Discussion

In anesthetized cats, both 1 and 2 exhibited agonist activity at muscarinic cholinoceptors and at α- and β-adrenoceptors. The muscle fasciculations and augmentation of soleus muscle twitches induced by 2 are typical characteristics of an anticholinesterase agent. In this capacity, 2 was approximately 200 times less potent than neostigmine. The possibility that the cardiac, blood pressure, and soleus defusion effects were produced indirectly, through release of the appropriate chemical transmitter by 1 and 2, is unlikely but cannot be excluded. Reduction in the tension and degree of fusion of the incomplete tetanic contractions of the soleus muscle is an

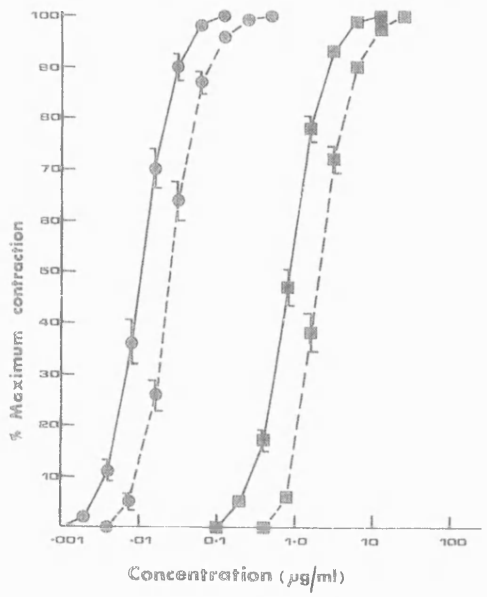


Figure 1. Log concentration–effect curves for carbachol (●) and 2 (■) on the guinea pig isolated ileum preparation. Solid lines represent the control curves before and broken lines the effect after 1×10^{-8} M atropine. The points plotted are the means of at least four separate experiments. Standard errors of the means are illustrated on the symbols ⊢■⊣ and ⊢●⊣.

effect mediated through stimulation of β_2-adrenoceptors.[10] The muscle is known to be sensitive to adrenaline but not noradrenaline;[10,11] hence, any indirect effect would necessitate the release of adrenaline from the adrenal medullae.

In vitro, both compounds possessed activity at cholinoceptors. In the chick biventer cervicis nerve-muscle preparation, both 1 and 2 produced neuromuscular blockade with accompanying contractures. Since the responses to exogenous carbachol were unaffected while those to exogenous acetylcholine were potentiated, it may be concluded that the blockade was of the depolarization type affecting principally the focally innervated fibers. This could be a consequence of anticholinesterase activity, such as was detected in biventer homogenates with 2. In the smooth muscle of the guinea pig ileum, 1 caused little effect, whereas 2 acted as a full agonist at muscarinic cholinoceptors.

The structural resemblance of the tetrahydroisoquinoline skeleton to dopamine has led to its use in studies of the conformational requirements for agonism at adrenoceptors.[8] In their study, Gray et al.[8] reported that 2 induced contraction of the guinea pig tracheal chain preparation at a concentration of 100 μg/mL. Adrenergic activity was assessed in other preparations over the concentration range 0.01–2000 μg/mL. Our studies have clearly demonstrated the existence of cholinergic effects for salsolinol (1) and 2 at concentrations well within the range used by Gray et al.[8] The closely related compound *N*-methyl-1,2,3,4-tetrahydroisoquinoline has also been shown to possess cholinergic activity.[12] The existence of this activity at cholinoceptors clearly complicates the use of such dopamine derivatives in the pursuit of the structural elucidation of adrenoceptors. These actions at cholinoceptors appear to have gone undetected in previous studies.

Experimental Section

(A) Pharmacological Methods. (1) Anesthetized Cat. The experiments were performed on ten adult cats of either sex which were anesthetized by the intraperitoneal injection of a mixture of α-choralose (80 mg/kg) and sodium pentobarbitone (6 mg/kg). The trachea was intubated and the cats were artificially ventilated by positive pressure at a frequency at 27–30 breaths/min and a stroke volume of 13 mL/kg of body weight. The general arterial blood pressure was measured from a cannulated carotid artery by means of a Statham (Model P23AC) pressure transducer. Heart rate was measured by means of a Grass (Model 7P4) tachograph triggered by the general arterial pulse.

The tendon of insertion of a soleus muscle was cut and attached to a Grass (Model FT03) force displacement transducer. Incomplete tetanic contractions of the soleus muscle were evoked by stimulating the peripheral end of the sciatic nerve, which had been dissected out and ligated deep in the thigh, at a frequency of 6–10 Hz for 1 s every 10 s. The method was identical with that described by Bowman and Nott.[10] In some experiments, maximal twitches of the soleus muscle were evoked by stimulating the sciatic nerve at a frequency of 0.1 Hz.

In all but one experiment, the cats were bilaterally vagotomized. All measurements were continuously recorded on a Grass six-channel curvilinear polygraph (Model 7). Drugs dissolved in acid–0.9% (w/v) saline (pH 4.5) were administered through a cannula inserted in either a femoral or brachial vein.

(2) Isolated Chick Biventer Cervicis Nerve Muscle Preparation. Biventer cervicis muscles from chicks aged 3–10 days were set up in Krebs–Henseleit solution at 37 °C and aerated with 5% CO_2 in oxygen. The method was identical with that described by Ginsborg and Warriner.[13]

(3) Isolated Guinea Pig Ileum. The isotonic contractions of segments of guinea pig ileum, set up in Tyrode[14] solution at 32 °C and bubbled with air, in response to several cholinoceptor agonists were recorded on a Washington (Model 400MD/2) pen recorder.

(4) Anticholinesterase Determination. The anticholinesterase activity of the two analogues was assessed by measuring the cholinesterase activity of homogenates of chick biventer cervicis muscle in the presence of each drug according to the colorimetric method of Ellman et al.,[15] as modified by Gandiha et al.[16] The absorbance changes were measured, using an SP 800 spectrophotometer, at a wavelength of 412 nm and a temperature of 32 °C.

(B) Chemical Methods. Melting points were recorded on a Kofler hot-stage apparatus and are uncorrected. IR and 60-MHz NMR spectra were recorded routinely for all steps and were consistent with assigned structures. Elemental analyses were performed by the Chemistry Departments of the Universities of Strathclyde and Manchester, U.K.

6,7-Dihydroxy-1-methyl-1,2,3,4-tetrahydroisoquinoline (1) Hydrobromide (Salsolinol Hydrobromide). 3,4-Dihydro-6,7-dimethoxy-1-methylisoquinoline (1.0 g, 0.005 M) prepared by routine methods[2] was dissolved in EtOH (20 mL), $NaBH_4$ (0.5 g, 0.014 M) was added, and the solution was left overnight. The solution was acidified with dilute HCl, rebasified with 20% NaOH, and extracted with $CHCl_3$ to give a viscous oil, which was dissolved in concentrated HBr (25 mL), refluxed for 4 h, evaporated, and azeotroped with a $CHCl_3$–EtOH mixture, and the residue was crystallized from ethanol–ether to give salsolinol (1) hydrobromide as small off-white prisms: mp 195–198 °C (lit.[2] 186–187 °C); MS *m/e* 179 ($C_{10}H_{13}NO_2$, 12%), 178 ($C_{10}H_{12}NO_2$, 18%), 164 ($C_9H_{10}NO_2$, 100%). Anal. ($C_{10}H_{14}NO_2Br$) C, H, N.

6,7-Dihydroxy-1,1-dimethyl-1,2,3,4-tetrahydroisoquinoline (2) Hydrobromide. 3,4-Dihydro-6,7-dimethoxy-1-methylisoquinoline (7.0 g, 0.034 M) prepared as usual was treated with benzyl bromide (6.0 g, 0.035 M) to give as a first crop 6.0 g of off-white solid, which was washed thoroughly with ether containing a little ethanol and then dried in vacuo. This salt (2.0 g, 0.005 M) was added to a solution of MeMgI in ether, which was prepared from MeI (4.0 g) and Mg (0.8 g). After 2 h of stirring, excess reagent was decomposed with NH_4Cl solution, and the aqueous layer was separated and extracted thoroughly with ether. The

combined ether layers were washed with water, dried, and evaporated to give a colorless gum (1.53 g). The gum (1.40 g) was taken up in ethanol (100 mL), and concentrated HCl (2 mL) and 10% Pd on C (0.2 g) were added and hydrogenated overnight at atmospheric pressure. Filtration and evaporation gave a pale-yellow oil (1.1 g). The oil (1.0 g) was dissolved in concentrated HBr (20 mL), and the solution was refluxed for 5 h. It was then evaporated, azeotroped twice with benzene–methanol so as to remove most of the water, and crystallized from 1-butanol to give small pale-gray plates that were washed with 1-butanol and ether and dried thoroughly. The yield at this stage was 0.70 g, but the product contained 1-butanol on crystallization that was removed by recrystallization from ethanol–ether to give small off-white prisms (mp 254–256 °C) containing no butanol (GLC): MS *m/e* 193 ($C_{11}H_{15}NO_2$, 2%), 178 ($C_{10}H_{12}NO_2$, 100%). Anal. ($C_{11}H_{16}NO_2Br$) C, H, N.

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References and Notes

(1) P. P. Laidlaw, *J. Physiol. (London)*, **40**, 480 (1910).
(2) P. N. Craig, F. P. Nabenhauer, P. M. Williams, E. Macko, and J. Toner, *J. Am. Chem. Soc.*, **74**, 1316 (1952).
(3) P. Holtz, *Pharm. Rev.*, **18**, 85 (1966).
(4) E. Yamato, M. Hirakura, and S. Sugasawa, *Tetrahedron, Suppl.*, **8**, 129 (1966).
(5) Y. Iwasawa and A. Kiyomoto, *Jpn. J. Pharmacol.*, **17**, 143 (1967).
(6) F. Fogelman and H. F. Grundy, *Br. J. Pharmacol.*, **38**, 416 (1970).
(7) J. Houston and I. W. Rodger, *Clin. Exp. Pharmacol. Physiol.*, **1**, 401 (1974).
(8) A. P. Gray, E. Reit, J. A. Ackerly, and M. Hava, *J. Med. Chem.*, **16**, 1023 (1973).
(9) D. W. Baldwin and E. Lesser, *Br. J. Pharmacol.*, **42**, 412 (1971).
(10) W. C. Bowman and M. W. Nott, *Br. J. Pharmacol.*, **38**, 37 (1970).
(11) W. C. Bowman and E. Zaimis, *J. Physiol. (London)*, **144**, 92 (1958).
(12) J. Z. Ginos, G. C. Cotzias, E. Tolosa, L. C. Tang, and A. LoMonte, *J. Med. Chem.*, **18**, 1194 (1975).
(13) B. L. Ginsborg and J. Warriner, *Br. J. Pharmacol. Chemother.*, **15**, 410 (1960).
(14) M. V. Tyrode, *Arch. Int. Pharmacodyn. Ther.*, **20**, 205 (1910).
(15) G. L. Ellman, K. D. Courtney, V. Andres, and R. M. Featherstone, *Biochem. Pharmacol.*, **7**, 88 (1961).
(16) A. Gandiha, A. L. Green, and I. G. Marshall, *Eur. J. Pharmacol.*, **18**, 174 (1972).

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Synthesis of 1-(Aminomethyl)-1,2,3,4-tetrahydroisoquinolines and Their Actions at Adrenoceptors in Vivo and in Vitro

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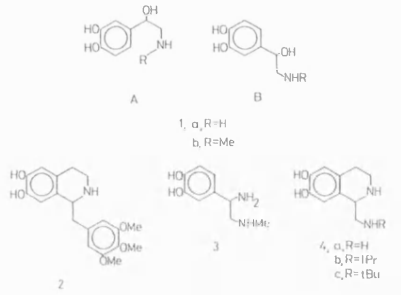
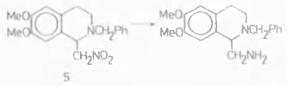
An improved synthesis of 1-(aminomethyl)-1,2,3,4-tetrahydroisoquinolines has been developed by using aluminum hydride reduction of 1-cyano-1,2,3,4-tetrahydroisoquinolines. Three 1-(aminomethyl)-6,7-dihydroxytetrahydroisoquinolines were tested for actions at β adrenoceptors in order to examine a proposed similarity between this series and the related phenylethanolamines. The aminomethyl, (isopropylamino)methyl, and (*tert*-butylamino)methyl derivatives all showed weak partial agonist activity at β adrenoceptors and the first also showed weak α adrenoceptor agonist activity in vivo. Their low potency implies that the catechol group of THIQ sympathomimetics, such as trimetoquinol, binds differently from that of the natural catecholamines. The protonation behavior of representative aminomethyl-THIQ's was investigated by pK_a measurement and ^1H and ^{13}C NMR, and the compounds were shown to be substantially monoprotonated, on the exocyclic nitrogen, at physiological pH.

Extensive work on the structural requirements for sympathomimetic activity in phenylethanolamines related to the natural transmitters epinephrine (1a) and norepinephrine (1b) has underlined the importance of the side-chain hydroxy group for high potency.[1] The discovery of potent β-adrenoreceptor stimulant activity[2] in the benzyltetrahydroisoquinoline derivative trimetoquinol (2) appeared to contradict the previous conclusions, since trimetoquinol and the phenylethanolamines could be partially superimposed (A and 2, Chart I). However, this requires the phenylethanolamine to adopt an unlikely cisoid conformation, and Brittain and co-workers made the suggestion[3] that the basic amino function of trimetoquinol might fulfill the receptor-binding role of the hydroxy group of the phenylethanolamine side chain (B and 2, Chart I). It has been reported[4] that the diamine (3) retains approximately one-twentieth of the potency of epinephrine (1b), which offers limited support to the concept that some NH/OH functions may be interchangeable. The proposal by Brittain and co-workers also requires the 3,4,5-trimethoxy group of trimetoquinol to fulfill the role of the amino group of epinephrine, which seems somewhat less likely. If this were so, then tetrahydroisoquinolines (4) should be effective sympathomimetics, and a recent patent claims "muscle-relaxant" activity among derivatives in this series.[5] We set out to improve the synthesis of some appropriately substituted analogues (4a–c) and to examine their actions at adrenoceptors.

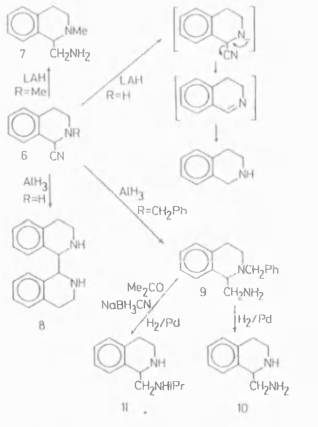
Chart I. Structural Relationships between Phenylethylamines and Tetrahydroisoquinolines



Scheme I



Scheme II



Chemistry. The first generally successful syntheses of the title class of compounds involved Bischler–Napieralski cyclization of *N*-phenethyl-2-phthalimidoacetamides, followed by reduction to the 1,2,3,4-tetrahydroisoquinoline and hydrazinolysis to remove the phthalyl protecting group.[6] A more attractive route (Scheme I) through the readily available 1-(nitromethyl)tetrahydroisoquinolines was reported to be unsuccessful,[7] resulting in elimination of nitromethane with a variety of reducing agents. In our hands, use of lithium aluminum hydride [or sodium bis(2-methoxyethoxy)aluminum hydride] gave yields of up to 25% of the desired product from the dimethoxy compound (5) (Scheme I). In seeking to improve on this, we noted that 1-cyano-2-methyl-1,2,3,4-tetrahydroisoquinoline (6, R = CH_3) was reported[9] to be reduced by lithium aluminum hydride to the 1-(aminomethyl)tetrahydroisoquinoline (7) in good yield (Scheme II). When we applied this method to the secondary amine (6, R = H), the cyano function was largely eliminated to give as major product

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(1) R. T. Brittain, C. M. Dean, and D. Jack, *Pharmacol. Ther., Part B*, 2, 423 (1976), provide a useful overview.

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Table I. β-Adrenoceptor Agonist Activities of THP and 4a–c in Guinea Pig, Isolated, Spontaneously Beating, Right Atrial Preparations

	intrinsic act.[a]		pD_2		drug $E_{max(50)}$/Iso[b] $E_{max(50)}$		
compd	mean	SEM	mean	SEM	mean	SEM	n
THP	0.94	0.01	6.96	0.09	26	2	4
4a	0.70	0.05	4.49	0.13	8.8×10^3	3.4×10^3	3
4b	0.42	0.03	5.05	0.09	2.7×10^3	0.4×10^3	3
4c	0.14	0.01	5.10	0.12	2.1×10^3	0.3×10^3	5

[a] Isoproterenol = 1. [b] Iso = isoproterenol.

Table II. β-Adrenoceptor Agonist Activities of THP and 4a–c in Reserpine-Pretreated, Guinea Pig, Isolated, Spontaneously Beating, Right Atrial Preparations

	intrinsic act.[a]		pD_2		drug $E_{max(50)}$/Iso[b] $E_{max(50)}$		
compd	mean	SEM	mean	SEM	mean	SEM	n
THP	0.95	0.01	7.05	0.03	38	8	4
4a	0.53	0.03	4.22	0.18	32.6×10^3	12.2×10^3	3
4b	0.59	0.06	5.18	0.06	4.4×10^3	1.3×10^3	3
4c	0.29	0.05	5.23	0.02	3.6×10^3	0.7×10^3	3

[a] Isoproterenol = 1. [b] Iso = isoproterenol.

1,2,3,4-tetrahydroisoquinoline itself, with only traces of the desired diamine. This could be attributed to initial removal of a proton from nitrogen by the basic reagent (Scheme II). However, use of the acidic reagent aluminum hydride was also unsuccessful, the major product (14%) being the unexpected 1,1′-bi(1,2,3,4-tetrahydroisoquinolinyl) (8). Similar products have been reported from the reduction of isoquinolines with aluminum amalgam.[24]

These problems were finally overcome by reduction of the *N*-benzyl derivative (6, R = CH_2Ph) with aluminum hydride, resulting in excellent yields of the aminomethyl compound (9), which was readily hydrogenolyzed to the model noradrenaline analogue (10). The model isoproterenol analogue (11) was prepared by reductive alkylation with acetone and sodium cyanoborohydride prior to debenzylation.

This process was applicable to the dimethoxy analogue (12, R = CH_3) as part of a route to the required 6,7-dihydroxy analogues (4, Scheme III), but a more convenient procedure was through the 6,7-bis(benzyloxy) derivatives 13 (R = CH_2Ph) and 14, where O- and N-debenzylation could be effected simultaneously at the final stage, to give 4a, or where intermediate alkylation was carried out, to give 4b. The *tert*-butyl derivative 4c was not available by this method, so a route was adopted that took advantage of the bulky *tert*-butyl group as a protecting function.

Scheme III

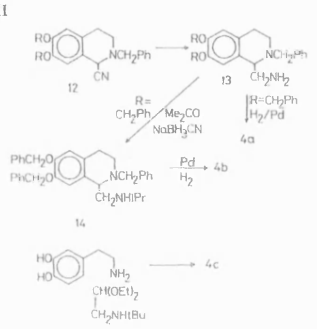


Table III. β-Adrenoceptor Antagonist Activities of 4a–c and THP in Guinea Pig, Isolated, Spontaneously Beating, Right Atrial Preparations

	pA_2		slope		
compd	mean	SEM	mean	SEM	n
4a	4.63	0.06	0.79	0.09	4
4b	4.84	0.01	0.99	0.02	5
4c	4.68	0.02	0.72[a]	0.03	6
THP	NA[b]				3

[a] Slope differs significantly from 1.0 (Student's t test, $p > 0.05$). [b] NA = no antagonism of isoproterenol responses up to 2.2×10^{-8} mol/L.

Thus, dopamine hydrochloride and (*tert*-butylamino)-acetaldehyde diethyl acetal were condensed in a Pictet–Spengler reaction to give the required product directly (Scheme III). With less bulky N-substituents, the amino group of the amino acetal would need to be protected, as in a recent report[6] where *N*-benzyloxycarbonyl functions were used.

Pharmacology. Preliminary tests showed that only the 6,7-dihydroxy analogues (4a–c) possessed any agonist or antagonist activity; the synthetic precursors and model compounds described in Schemes I–III were inactive. The detailed examination was therefore confined to compounds 4a–c, and the results are summarized in Tables I–VI. Isoproterenol was used as standard throughout, but the compounds were also compared to tetrahydropapaveroline (THP) as a representative 6,7-dihydroxytetrahydroisoquinoline.

(2) E. Yamato, M. Hirakura, and S. Sugasawa, *Tetrahedron, Suppl.*, 8(1), 129 (1966). Y. Iwasawa and A. Kiyomoto, *Jpn. J. Pharmacol.*, 17, 143 (1967). J. Houston and I. W. Rodger, *Clin. Exp. Pharmacol. Physiol.*, 1, 401 (1974). The subject has recently been reviewed: D. Beaumont and R. D. Waigh, *Prog. Med. Chem.*, 18, 45 (1981).
(3) R. T. Brittain, D. Jack, and A. C. Ritchie, *Adv. Drug Res.*, 5, 197 (1970).
(4) G. Lehmann and L. O. Randall, *J. Pharmacol. Exp. Ther.*, 93, 114 (1948).
(5) T. Kishimoto, H. Kochi, and M. Kato, German Offen. 2549901 (1976). T. Kishimoto, I. Ueda, and M. Kato, British Patent 1553230 (1979).
(6) H. J. Harwood and T. B. Johnson, *J. Am. Chem. Soc.*, 55, 4178 (1933). See also L. G. Humber, *Can. J. Chem.*, 49, 857 (1971). G. Redeuilh, B. Marcot and C. Viel, *Bull. Soc. Chim. Fr.*, 850 (1975). Y. Takeo, *Yakugaku Zasshi*, 79, 1003 (1959); *Chem. Abstr.*, 54, 5679e (1960).
(7) R. D. Haworth and W. H. Perkin, Jr., *J. Chem. Soc.*, 1434 (1925).
(8) H. C. Brown and N. M. Yoon, *J. Am. Chem. Soc.*, 88, 1464 (1966).
(9) H. Boehme and K.-P. Stoecker, *Chem. Ber.*, 105, 1578 (1972).

Table IV. β-Adrenoceptor Agonist Activities of 4a-c and THP in Guinea Pig, Isolated, Carbachol-Contracted Tracheal Preparations

	intrinsic act.[a]		pD_2		drug $E_{max(50)}$/Iso $E_{max(50)}$		
compd	mean	SEM	mean	SEM	mean	SEM	n
THP	0.62	0.04	6.45	0.09	6	1	4
4a	0.20[b]	0.02	3.44[b]		2.5×10^{4}[c]	0.6×10^{4}	4
4b	0.06[b]	0.02	3.49[b]		ND		3
4c	0.18[b]	0.01	3.51[b]		2.0×10^{4}[c]	0.6×10^{4}	5

[a] Isoproterenol = 1. [b] The maximum concentration used was 100 μg/mL. This did not give maximum responses. Therefore, estimates of intrinsic activities are low, the values merely reflecting the response to 100 μg/mL. Likewise, pD_2 values were not determined. Values cited here are −log (molar equivalent of 100 μg/mL). [c] Ratio determined at concentrations required to produce 15% of maximum isoproterenol response.

All four drugs (4a–c) and THP increased atrial rate, but maximum effects were less than those of isoproterenol. These effects were blocked by propranolol (1 μmol/L). Because of differences in intrinsic activity between the agents and isoproterenol, activity ratios rather than potency ratios were used in characterizing the drugs. The activity ratio is the ratio of the molar concentration of the drug required to produce 50% of its maximum effect [$E_{max(50)}$] and the molar concentration of isoproterenol required to produce its $E_{max(50)}$ in the same experiment. Intrinsic activities, pD_2 values, and activity ratios are shown in Table I. Table II shows similar results from reserpine pretreated guinea pigs.

Antagonistic Action. All drugs, with the exception of THP, shifted cumulative concentration–effect curves of isoproterenol to the right without affecting their maximum responses. Atrial rate was increased by the concentrations of the drugs required for antagonistic action; the increased rate was sustained over the 15-min contact period. pA_2 values and slopes of the relationship between log (dose ratio minus 1) and negative log (molar antagonist concentration) from all experiments are shown in Table III.

Guinea Pig Trachea. Agonistic Action. THP produced the greatest relaxing activity, and the maximum effect was obtained within the concentration range studied. The analogues 4a–c were very weak agonists, and with the highest concentration used (100 μg/mL), maximum effects were not obtained. Thus, pD_2 values, intrinsic activities, and activity ratios of the drugs could not be calculated, but approximations of the values are shown in Table IV. The agonist effects were blocked by propranolol (1 μmol/L).

Antagonistic Action. All of the compounds under test, with the exception of THP, shifted cumulative concentration–effect curves of isoproterenol to the right without affecting their maximum effects. With the lowest concentration of the drugs (4c, 31; 4b, 32; 4a, 36 μmol/L), generally no relaxation in response to the drugs per se occurred. With concentrations 3 and 10 times higher, some relaxation occurred. pA_2 values and the slopes of the relationship between log (dose ratio minus 1) and negative log (molar antagonistic concentration) are shown in Table V.

Anesthetized Cat Cardiovascular System. All three analogues (4a–c) caused tachycardia in anesthetized and bilaterally vagotomized cats, but maximum responses were less than that produced by isoproterenol. The effect was blocked by prior administration of propranolol (100 μg/kg iv). The maximum depression of pressure caused by 4b and 4c was achieved at doses that just produced a maximum increase in heart rate. Again, the maximum depression was less than that seen with isoproterenol.

In contrast to the other agents, 4a produced a pronounced pressor response; this was abolished by prior administration of phenoxybenzamine (3 mg/kg). These

Table V. β-Adrenoceptor Antagonist Activities of 4a-c and THP in Guinea Pig, Isolated, Carbachol-Contracted Tracheal Preparations

	pA_2		slope		
compd	mean	SEM	mean	SEM	n
4a	4.66	0.08	0.58[a]	0.08	4
4b	5.05	0.05	0.57[a]	0.04	4
4c	5.76	0.69	0.44[a]	0.08	6
THP	NA[b]				3

[a] Slope differs significantly from 1.0 (Student's t test, $p > 0.05$). [b] NA = no antagonism of isoproterenol responses in concentrations up to 2.2×10^{-5} mol.

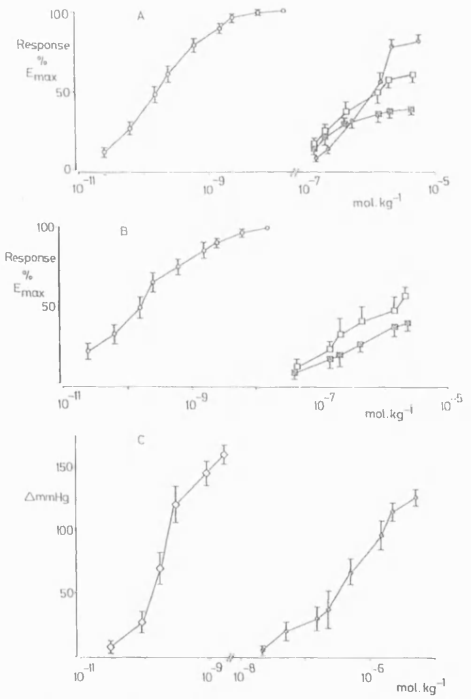


Figure 1. Log concentration–effect curves for isoproterenol (○), 4a (⊗), 4b (□), 4c (▦), and norepinephrine (◇) in chloralose-anesthetized, bilaterally vagotomized cats: (A) positive chronotropic effect, (B) decrease in diastolic blood pressure, (C) increase in systolic blood pressure. Results in A and B are expressed in terms of percent maximum response (E_{max}) produced by isoproterenol and in C in terms of increase in pressure (mmHg). Individual points show mean plus or minus SEM responses from three to six experiments.

Table VI. Intrinsic Activity Ratios and Equieffective Dose Ratios of 4a–c in Increasing (HR) and Reducing Diastolic Blood Pressure (DBP) in Chloralose Anaesthetized, Bilaterally Vagotomized Cats

	HR				DBP				
	α[a]		activity ratio[b]		α		act. ratio		
compd	mean	SEM	mean	SEM	mean	SEM	mean	SEM	n
4a	0.80	0.06	1.27×10^{4}	0.42×10^{4}			pressor		5
4b	0.60	0.05	0.17×10^{3}	0.05×10^{3}	0.56	0.07	2.20×10^{3}	0.81×10^{3}	5
4c	0.38	0.03	0.09×10^{3}	0.01×10^{3}	0.34	0.05	1.05×10^{3}	0.72×10^{3}	5

[a] Isoproterenol = 1. [b] For calculation of the activity ratio, the molar concentration of the compound required to produce 50% of its own maximum response was divided by the molar concentration of isoproterenol required to produce 50% of isoproterenol maximum response in the same experiment.

results are illustrated in dose–response form in Figure 1A–C. Table VI shows the intrinsic activities and activity ratios of the compounds on heart rate and diastolic blood pressure.

Discussion

The results show that the compounds are weak partial agonists at β adrenoceptors. Thus, in guinea pig isolated preparations, they exerted a positive chronotropic effect on atria, and all relaxed tracheal smooth muscle; these effects were antagonized by the β-adrenoceptor antagonist propranolol. Their antagonistic action was manifested by their ability to block response to isoproterenol. The sympathomimetic effects appear to be due to a direct action at β adrenoceptors, since the positive chronotropic effect was unaffected by reserpine pretreatment.

In terms of drug–receptor interactions, the affinity of a partial agonist for receptors is related to its pA_2 or pD_2 values. For their antagonistic action on atria, data on the compounds under test conform with criteria for competitive interactions; the slopes of the plot between negative log (dose ratio minus 1) and negative log (molar antagonist concentration) do not differ significantly from unity. However, the data from the experiments on tracheae are inconsistent with competitive interactions at a single receptor site; the slopes of the plots are only around 0.5. Thus, the pA_2 values from atrial experiments provide a more appropriate measure of affinity than those obtained from experiments on tracheae. For agonistic potency, only the pD_2 values from atria could be determined, since the activity of the compounds on the tracheae was very low; at the highest concentration used, maximum relaxation was not obtained.

The affinities of the compounds did not change with increasing size of aminoalkyl loading at the 1-position but increased greatly when the substituent at the 1-position was a 3,4-dihydroxybenzyl group (i.e., THP); this is more closely related to, and can rotate as freely as, the catechol moiety of catecholamines. It is still uncertain which of the two dihydroxy groups, if either, combines with the catechol site of the physiological receptor. Yamato et al. found[2] that 1-[3,4-(ethylenedioxy)benzyl]-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinolines possessed agonist activity considerably greater than that of 1-(3,4-dihydroxybenzyl)-6,7-(ethylenedioxy)-(1,2,3,4-tetrahydroisoquinolines and suggested that the two hydroxy groups in the 6- and 7-position were essential for the activity.

The high agonist activity of trimetoquinol (2) and the deleterious effect on potency of modifications to the 6,7-dihydroxy substitution pattern[10] emphasize the importance of the catechol groups in this position for receptor binding and suggest that the extra catechol moiety of THP is finding an alternative binding site, which is not the same as the ammonium binding site of the phenethanolamines, as evidenced by the present results.

In the present experiment, because of dose limitations the activities of the compounds could not be determined in the trachea. However, it seems that true values were higher than those shown in Table IV, since in occasional experiments when higher concentrations were used, they produced further relaxation. In contrast, the activities of all compounds were easily determined in the atria. With this limited information on tracheal activities, some inference can be made about selectivity for β adrenoceptors in this tissue and in the atria.

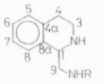
In terms of selectivity for bronchial vs. cardiac β adrenoceptors, it has been shown for phenylethanolamines that selectivity can be achieved by different means. Thus with *tert*-butyl substitution at the amino group, *N*-*tert*-butylnorepinephrine is found to be more selective than isoproterenol.[1]

The present results agree in some respects with the above report. With its 1-[(*tert*-butylamino)methyl] substitution, 4c showed some degree of selectivity, in terms of a higher intrinsic activity in tracheae than in atria, although in terms of the ratio of the potency with that of isoproterenol, it is 10 times less selective for tracheal β adrenoceptors (compare ratios in Tables I and IV). It thus differs from selective β-adrenoceptor agonists, such as salbutamol, soterenol, terbutaline, and carbuterol, which show a marked selectivity for tracheal β adrenoceptors but have high intrinsic activity in atria. The question arises whether a compound with a pharmacological profile comparable to 4c with higher intrinsic activity in tracheae than in atria and in spite of low potency vis a vis isoproterenol in tracheae could show a functional selectivity for bronchial smooth-muscle relaxation over cardiac excitation. It seems, however, that unless a molecular modification can be made to enhance their potency, such compounds are unlikely to be useful as bronchodilators in clinical practice. Indeed, the β-adrenoceptor antagonist actions of the existing compounds would presumably be clinically undesirable in asthmatics who depend on endogenous catecholamines for maintenance of adequate caliber of airways.

Results obtained with 4a–c on blood pressure in anesthetized cats show that the in vivo β-adrenoceptor agonist activity of the compounds is enhanced by an increase in size of the 1-substituent. Thus, with its 1-(aminomethyl) substituent, 4a possessed a strong pressor action that was prevented by the α-adrenoceptor antagonist phenoxybenzamine, but the compounds with large substituents were depressor agents, with a weak pressor action only manifested in the presence of β-adrenoceptor blockade. The enhancement in β-adrenoceptor agonist selectivity with increasing size of an N-substituent in the catecholamine molecule is well documented[1] and implies

(10) K. Ikezawa, H. Takenaga, M. Sato, H. Nakajima, and A. Kiyomoto, *Jpn. J. Pharmacol.*, 27, 537 (1977). M. Sato, I. Yamaguchi, and A. Kiyomoto, *Jpn. J. Pharmacol.*, 17, 153 (1967). K. Ikezawa, H. Takenaga, M. Sato, N. Itoh, H. Nakajima, and A. Kiyomoto, *Yakugaku Zasshi*, 74, 441 (1978); *Chem. Abstr.*, 89, 190806*a* (1978).

Table VII. ^{13}C NMR Spectral Data for 1-(Aminomethyl)-1,2,3,4-tetrahydroisoquinolines



10, R=H

11, R= —CH(CH3)2

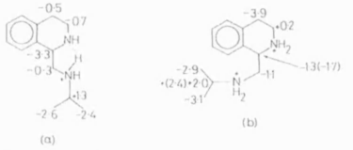
	$CDCl_3$		H_2O containing 15–20% v/v D_2O			
solvent	THIQ[d] free base	10 free base	10 free base	11 free base	11·HCl monoprotonated	11·2HCl diprotonated
C8a	134.8	137.0 (s)[a]	136.4 (s)[a]	137.3 (s)[a]	134.0 (s)[a]	128.1 (s)
C4a	136.1	136.1 (s)[a]	135.9 (s)[a]	136.5 (s)[a]	136.3 (s)[a]	133.0 (s)
C5	129.2	129.4 (d)	130.3 (d)	130.3 (d)	130.6 (d)	130.5 (d)[a]
C8	126.1	126.2 (d)[b]	127.8 (d)[b]	127.7 (d)[b]	128.4 (d)[b]	130.3 (d)[a]
C7	125.9	126.1 (d)[b]	127.3 (d)[b]	127.5 (d)[b]	127.4 (d)[b]	128.5 (d)[b]
C6	125.6	125.9 (d)[b]	127.1 (d)[b]	127.1 (d)[b]	127.4 (d)[b]	127.9 (d)[b]
C1	48.2	57.6 (d)	56.9 (d)	55.0 (d)	51.7 (d)	53.7 (d)[c]
C10				51.3 (d)	52.6 (d)	53.3 (d)[c]
C9		46.4 (t)	45.5 (t)	49.0 (t)	48.7 (t)	47.9 (t)
C3	43.8	40.3 (t)	39.9 (t)	39.5 (t)	38.8 (t)	39.7 (t)
C4	29.1	29.8 (t)	28.8 (t)	29.0 (t)	28.5 (t)	25.1 (t)
C11				22.1 (q)	19.7 (q)	19.0 (q)
C12				21.9 (q)	19.3 (q)	19.0 (q)

[a–c] Indicate assignments may be interchanged. multiplicities [singlet (s), doublet (d), triplet (t), and quartet (q)] were obtained from SFORD spectra. [d] From ref 13.

that the exocyclic amino function of 4a–e is binding in a similar way to the amino function of phenethanolamines. If this is accepted, the low potency of 4a–c implies that the phenethanolamine receptor does not bind readily to the 6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline nucleus, at least when presented in a way that allows the exocyclic amino group to bind.

Some doubt existed concerning the protonation behavior of the diamines; we therefore sought to exclude this as a reason for lack of potency. Double protonation at physiological pH would lead to repulsion between the charged groups and the adoption of a conformation different from that suggested for active catecholamines.[11] Monoprotonation could occur on either nitrogen, with one of the alternatives bearing markedly reduced resemblance to the monobasic phenylethanolamines. Determination of pK_a values for the simple diamines (10 and 11) by potentiometric titration established that both would be substantially monoprotonated at physiological pH (pK_a values for 10 were 8.9 and 5.7; for 11 they were 9.2 and 5.4). Correction to 37 °C did not change this conclusion. To determine whether protonation was selective, i.e., which if either nitrogen was protonated predominantly first, we examined ^1H and ^{13}C NMR spectra of the bases 10 and 11 and the monohydrochloride and dihydrochloride of 11. Overlapping of peaks in the ^1H NMR spectra reduced the useful information to the observation that C(1) H underwent downfield shifts of 0.18 and 1.11 ppm on mono- and diprotonation, respectively, indicating that protonation occurred preferentially on the exocyclic nitrogen. More extensive information was obtained from ^{13}C NMR spectra, although protonation shifts in ^{13}C NMR spectra of polyamines are complex.[12]

Chart II. (a) Monoprotonation and (b) Diprotonation Shifts[a] in ^{13}C NMR Spectra of 1-(Aminomethyl) 1,2,3,4-tetrahydroisoquinolines[b]



[a] In parts per million. [b] Values in parentheses are uncertain peak assignments.

^{13}C NMR chemical shifts for compounds 10 and 11 are given in Table VII. Data for compound 10 were obtained in deuteriochloroform to facilitate comparison with published assignments for 1,2,3,4-tetrahydroisoquinoline,[13] although, in fact, there were only slight differences between spectra in chloroform and water. To a certain extent the assignments are arbitrary, and with a few significant exceptions, the protonation shifts are uncertain. Fortunately, a sufficient number of the peaks can be assigned to allow useful conclusions to be drawn; the protonation shifts resulting from the assignments in the table are expressed diagrammatically (Chart II).

The salient conclusions drawn from published amine ^{13}C NMR data are that (a) β carbons invariably show upfield protonation shifts while γ and δ carbons show small upfield shifts and (b) protonation shifts for α carbons vary from 2.5 ppm upfield to 8.8 ppm downfield.[12] It is apparent that the shifts in Chart II are consistent with monoprotonation occurring substantially on the exocyclic nitrogen. Of particular significance are the shifts of the isopropyl methyl carbons, which show a substantial upfield shift on monoprotonation and a much smaller extra shift on di-

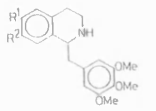
(11) A. M. Anderson, *Acta. Chem. Scand., Ser. B*, 29, 239 and 871 (1975). M. Mathew and G. J. Palenik, *J. Am. Chem. Soc.*, 93, 497 (1971). N. C. Stephenson and J. P. Beale, *J. Pharm. Pharmacol.*, 24, 277 (1972). J. E. Forrest, R. A. Heacock, and T. P. Forrest, *J. Pharm. Pharmacol.*, 22, 512 (1970). R. R. Ison, P. Parkington, and C. C. K. Roberts, *Mol. Pharmacol.*, 9, 756 (1973). C. Petrongola, J. Tomasi, B. Macchia, and F. Macchia, *J. Med. Chem.*, 17, 501 (1974). J. M. George, L. B. Kier, and J. R. Hoyland, *Mol. Pharmacol.*, 2, 328 (1971).

(12) J. E. Sarneski, H. L. Surprenant, F. K. Molen, and C. N. Reilley, *Anal. Chem.*, 47, 2116 (1975).

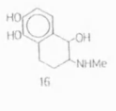
(13) D. W. Hughes, H. L. Holland, and D. B. MacLean, *Can. J. Chem.*, 54, 2252 (1976). The assignments given by another group may be in error: S. P. Singh, S. S. Parmar, V. I. Stenberg, and S. A. Farnum, *J. Heterocycl. Chem.*, 15, 541 (1978).

protonation, and C(4), which only shows a significant upfield shift on diprotonation. In both cases, the carbons are β to the proposed protonation site and unambiguously assignable.

Unambiguous information as to the conformation adopted by non-, mono-, and diprotonated species was not obtained. Solution of the Karplus equation for the coupling constants for C(1) H with the adjacent CH_2 group gave sets of dihedral angles from which two possible conformations for each species could be constructed. The conformations depicted in Chart II are permitted, and it is conceivable that the conformation of the monoprotonated molecule could be maintained by intramolecular hydrogen bonding. Such a conformation places the aryl and two amino functions in the same relative spatial arrangement as the aryl, amino, and hydroxy functions of arylethanolamines in what is considered to be their active conformation.[11] Further, such an arrangement closely resembles the relatively rigid and highly potent[14] tetrahydronaphthalenes, such as **16**. It appears that the diamines **4a–c** are closer in structure to active compounds in other series than is trimetoquinol. Their lack of potency compared to trimetoquinol indicated that the latter may bind to the receptor in a quite different manner from other β-adrenoceptor stimulants. For a fuller discussion, see Beaumont and Waigh.[2] The lack of potency of the two salbutamol analogues (**15a,b**) tends to support this hypothesis, although no pharmacological details were given.[15] Further work in this direction is being carried out.



15, a, R^1=OH, R^2=CH_2OH
b, R^1=CH_2OH, R^2=OH



16

Experimental Section

Chemical Methods. Melting points were obtained on a Reichert hot-plate apparatus and are corrected. Infrared spectra were obtained on a Perkin-Elmer 237 spectrophotometer from liquid films between sodium chloride plates or, for solids, from potassium chloride disks and are quoted in wavenumbers (reciprocal centimeters). Nuclear magnetic resonance spectra were obtained at 60-MHz on a Perkin-Elmer R12B instrument, at 90-MHz on a Perkin-Elmer R32 spectrometer, or at 80-MHz on a Bruker WP80 machine. The latter machine was also used to obtain ^{13}C NMR spectra at 20 MHz. Unless stated otherwise, deuteriochloroform was used as solvent with tetramethylsilane as internal standard. Chemical shifts are quoted in parts per million downfield from Me_4Si on the δ scale. Mass spectra were obtained on an AE1 MS12 spectrometer and are quoted as mass per charge ratio and relative abundance (percent) for the most abundant ions. Microanalyses was carried out on a Perkin-Elmer 240 CHN analyzer and were within 0.4% of theory. Compounds are only described as hemihydrates, where spectral data indicated a high degree of purity, and repeated recrystallization, thorough drying and repeated analyses, gave consistent results. Aluminum hydride was generated and used according to Brown and Yoon,[8] and reactions were worked up by the method of Hey and Palluel.[16]

2-Benzyl-6,7-dimethoxy-1-(nitromethyl)-1,2,3,4-tetrahydroisoquinoline (5). Benzyl bromide (17 mL, 142 mmol) was added to a solution of 6,7-dimethoxy-3,4-dihydroisoquinoline (13 g, 68 mmol) in dry benzene (80 mL), and the mixture was stirred and boiled under reflux for 2 h. The mixture was cooled, and the precipitate was filtered off, washed with ether, dried, and recrystallized from methanol–ether to give the *N*-benzyldihydroisoquinolinium bromide (23 g, 93%): mp 186–188 °C (from *N,N*-dimethylformamide–ethanol–ether) (lit.[17] mp 192–195 °C dec); IR 1645 cm^{-1}; ^1H NMR δ 3.0–3.4 (2 H, t, J_{app} = 8 Hz, $ArCH_2CH_2$), 3.7–4.1 (2 H, m, $CH_2CH_2N^+$), 3.84 and 3.94 (3 H, s, OCH_3), 5.47 (2 H, s, NCH_2Ph^+), 6.91 (1 H, s, Ar*H*), 7.2–7.65 (6 H, m, Ar*H*), 10.33 (1 H, s, C_1 H).

A solution of nitromethane (2.2 mL, 41 mmol) and potassium hydroxide (3 g, 54 mmol) in methanol (30 mL) was added to a solution of 2-benzyl-6,7-dimethoxy-3,4-dihydroisoquinolinium bromide (9.8 g, 27 mmol) in methanol (50 mL). After standing for 1 h, the mixture was evaporated to dryness, dissolved in water, neutralized with dilute hydrochloric acid, rebasified with sodium bicarbonate, and extracted with chloroform to give the **1-(nitromethyl)-1,2,3,4-tetrahydroisoquinoline** (7.8 g, 84%): ^1H NMR δ 2.1–3.4 (4 H, m, $ArCH_2CH_2N$), 3.73 (2 H, NCH_2Ph), 3.78 and 3.81 (3 H, s, OCH_3), 4.2–4.8 [3 H, m, $ArCH(N)CH_2NO_2$], 6.53 and 6.61 (1 H, s, Ar*H*), 7.24 (5 H, s, Ar*H*). A portion purified from acetone had mp 104–105 °C; IR 1550 and 1360 cm^{-1}. Anal. ($C_{19}H_{22}N_2O_4$) C, H, N.

Reduction of 2-Benzyl-6,7-dimethoxy-1-(nitromethyl)-1,2,3,4-tetrahydroisoquinoline. 1. With Lithium Aluminum Hydride: "Normal Addition". A solution of the 1-(nitromethyl) derivative (1.9 g, 5.5 mmol) in dry tetrahydrofuran (50 mL) was added to a stirred suspension of lithium aluminum hydride (0.6 g, 16 mmol) in dry tetrahydrofuran (30 mL), and the mixture was boiled under reflux for 1 h. Workup gave a mixture of products, which were dissolved in dry ether and dripped into ethereal hydrogen chloride. The solid was collected and recrystallized from methanol to give 1-(aminomethyl)-2-benzyl-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline dihydrochloride (0.35 g, 16%), which was converted to the free base and gave spectral data identical with those of an authentic sample (see below).

(2) With Lithium Aluminum Hydride: "Reverse Addition". Lithium aluminum hydride (1 g, 26 mmol) was placed in a soxhlet thimble. The 1-(nitromethyl) derivative (2.8 g, 8.3 mmol) in dry tetrahydrofuran (100 mL) was placed in the flask beneath, boiled under reflux for 1 h, and worked up to give 2-benzyl-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline (2 g, 85%). ^1H NMR δ 2.70 (4 H, br s), 3.48 and 3.60 (2 H, s), 3.70 and 3.73 (3 H, s), 6.40 and 6.53 (1 H, s), 7.1–7.5 (5 H, m). A portion crystallized from toluene–petroleum ether (bp 60–80 °C) had mp 68–69 °C (lit.[17] mp 88–89 °C). The spectral data and melting point were identical with those of an authentic sample prepared by the sodium borohydride reduction of 2-benzyl-6,7-dimethoxy-3,4-dihydroisoquinolinium bromide.

(3) With Sodium Bis(2-methoxyethoxy)aluminum Hydride. A solution of the 1-(nitromethyl) derivative (2.1 g, 6 mmol) in dry benzene (60 mL) was dripped into a stirred, boiling solution of Red-Al (6 mL), containing approximately 16 mmol of sodium bis(2-methoxyethoxy)aluminum hydride in dry benzene (40 mL), and the mixture boiled under reflux for 1 h. Dilute sodium hydroxide (40 mL) was added, and the mixture was stirred vigorously for 1 h. The organic layer was separated and evaporated to give a mixture of products, which were dissolved in ethanol and dripped into ethereal hydrogen chloride. The precipitate was collected and purified from 2-propanol to give 1-(aminomethyl)-2-benzyl-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline dihydrochloride (0.6 g, 25%), which was converted to the free base and gave spectral data identical with those of an authentic sample.

1-Cyano-1,2,3,4-tetrahydroisoquinoline (6, R = H). A solution of potassium cyanide (2.5 g, 38 mmol) in water (20 mL) was added to a solution of 3,4-dihydroisoquinoline (4 g, 30 mmol) in water (20 mL) previously acidified with dilute hydrochloric acid. Extraction with ether gave the nitrile (4.7 g, 98%): IR 3340 and 2220 cm^{-1}; ^1H NMR δ 2.21 (1 H, s, exchangeable), 2.5–3.4 (4 H, m), 4.94 (1 H, s), 7.13 (4 H, s). The hydrochloride salt had mp 158–161 °C (lit.[18] mp 160–162 °C).

(14) M. Nishikawa, M. Kanno, H. Kuriki, H. Sugihara, M. Motohashi, K. Itoh, O. Miyashita, Y. Oka, and Y. Sanno, *Life. Sci.*, **16**, 305 (1975). Y. Oka, M. Motohashi, H. Sugihara, O. Miyashita, K. Itoh, M. Nishikawa, and S. Yurugi, *Chem. Pharm. Bull.*, **25**, 632 (1977).

(15) S. F. Dyke, A. W. C. White, and D. Hartley, *Tetrahedron*, **29**, 857 (1973).

(16) D. H. Hey and A. L. Palluel, *J. Chem. Soc.*, 2926 (1957).

(17) J. H. Chapman, P. G. Holton, A. C. Ritchie, T. Walker, G. B. Webb, and K. D. E. Whiting, *J. Chem. Soc.*, 2471 (1962).

Reduction of 1-Cyano-1,2,3,4-tetrahydroisoquinoline. 1. With Lithium Aluminum Hydride. 1-Cyano-1,2,3,4-tetrahydroisoquinoline (4.6 g, 29 mmol) was reduced with lithium aluminum hydride (3 g, 89 mmol) in ether to give a mixture of products. The crude products were distilled to give 1,2,3,4-tetrahydroisoquinoline (1.6 g, 41%): bp 104–106 °C (0.8 mmHg); IR 3250 cm^{-1}; ^1H NMR δ 1.42 (1 H, s), 2.5–3.2 (4 H, m), 3.85 (2 H, s), 6.7–7.3 (4 H, m). The hydrochloride salt had mp 195–196 °C (from ethanol) (lit.[19] mp 197–198 °C). The residue after distillation was dissolved in ethanol and dripped into an ethereal solution of oxalic acid. The precipitate was collected and purified from ethanol to give 1-(aminomethyl)-1,2,3,4-tetrahydroisoquinoline dioxalate (0.2 g, 2%): spectral data identical with those of an authentic sample.

(2) With Aluminum Hydride. A solution of 1-cyano-1,2,3,4-tetrahydroisoquinoline (4.2 g, 27 mmol) in dry ether (50 mL) was dripped into a cooled, stirred solution of aluminum hydride (262 mmol) in dry ether (400 mL) and stirred overnight to give a mixture of products. The mixture was dissolved in ethanol and dripped into an ethereal solution of oxalic acid. The precipitate was collected and boiled in an ethanol–methanol mixture. After the mixture cooled, the precipitate was filtered off and recrystallized from water to give 1-(aminomethyl)-1,2,3,4-tetrahydroisoquinoline dioxalate (0.2 g, 2%): melting point and spectral data identical with those of an authentic sample. The filtrate (ethanol–methanol) was evaporated to dryness, and the residue was recrystallized from methanol to give 1,1′-bi-(1,2,3,4-tetrahydroisoquinolyl) (8) dioxalate (0.8 g, 14%): mp 190–192 °C; ^1H NMR (D_2O; external Me_4Si) δ 2.8–3.6 (8 H, m, 2 $ArCH_2CH_2NH_2^+$), 4.25 (2 H, s, 2 $ArCHNH_2^+$), 7.1–7.5 (8 H, m, Ar*H*); MS, *m/e* 133 (53), 132 (84), 104 (100). No molecular ion was observed; $C_{18}H_{20}N_2$ requires *m/e* 264. Anal. ($C_{22}H_{24}N_2O_8$) C, H, N. The free base gave IR 3260 cm^{-1}.

1-(Aminomethyl)-1,2,3,4-tetrahydroisoquinoline (10). Benzyl bromide (28 mL, 235 mmol) was added to a solution of 3,4-dihydroisoquinoline (18.7 g, 143 mmol) in dry ether (300 mL), and the mixture was stirred overnight at ambient temperature. The precipitate, which was hygroscopic, was filtered off to give the **dihydroisoquinolinium bromide** (31.5 g, 73%), which after drying on a rotary evaporator was obtained as a golden oil: ^1H NMR δ 3.20 (2 H, t, J_{app} = 8 Hz, $ArCH_2$), 4.05 (2 H, t, J_{app} = 8 Hz, $CH_2CH_2N^+$), 5.60 (2 H, s, NCH_2Ph^+), 7.0–8.15 (9 H, m, Ar*H*), 10.55 (1 H, s, C_1H). A portion crystallized from aqueous ethanol–ether had mp 60–72 °C; IR 3400 (H_2O), 1655.

A solution of potassium cyanide (1 g, 15 mmol) in water (20 mL) was added to a solution of 2-benzyl-3,4-dihydroisoquinolinium bromide (3.6 g, 12 mmol) in water (20 mL) and extracted with ether to give 2-benzyl-1-cyano-1,2,3,4-tetrahydroisoquinoline (2.8 g, 96%): ^1H NMR δ 2.5–3.3 (4 H, m), 3.78 (2 H, s), 4.59 (1 H, s), 6.8–7.5 (9 H, m). A portion recrystallized from petroleum ether (bp 60–80 °C) had mp 82–84 °C (lit.[9] mp 82 °C); IR 2220 cm^{-1}. The nitrile (5.7 g, 23 mmol) dissolved in dry ether (250 mL) was added to aluminum hydride (131 mmol) in dry ether (200 mL) and stirred overnight to give 1-(aminomethyl)-2-benzyl-1,2,3,4-tetrahydroisoquinoline (9; 5.1 g, 88%): IR 3360 cm^{-1}; ^1H NMR δ 1.35 (2 H, s, NH_2), 2.5–3.3 (6 H, m, $ArCH_2CH_2N$ and $CHCH_2NH_2$), 3.55 [1 H, t, J = 6 Hz, $ArCH(CH_2)N$], 3.56 and 3.80 (1 H, d, J_{gem} = 13.5 Hz, CH of NCH_2Ph), 6.8–7.4 (9 H, m, Ar*H*).

The tetrahydroisoquinoline (9; 5 g, 20 mmol) in 96% ethanol (150 mL) was hydrogenated at atmospheric pressure over 5% palladium on charcoal (1 g). Hydrogen uptake was slow, and after 24 h only 75% of theoretical uptake had occurred. The mixture was heated at 50 °C for a further 12 h. The mixture was filtered through Kieselguhr, and the filtrate was evaporated to give 1-(aminomethyl)-1,2,3,4-tetrahydroisoquinoline (10; 3 g, 93%): IR 3260 cm^{-1}; ^1H NMR δ 1.91 (3 H, s, NH_2 and NH), 2.55–3.20 (6 H, m, $ArCH_2CH_2NH$ and $CHCH_2NH_2$), 3.87 [1 H, t, J = 6 Hz, $ArCH(CH_2)N$], 7.05 (4 H, s, Ar*H*). The **dioxalate salt** had mp 187–189 °C (from aqueous methanol); ^1H NMR (D_2O; DSS) δ 3.17 (2 H, m $ArCH_2CH_2$), 3.5–3.8 (4 H, m, $CH_2CH_2NH_2^+$ and $CHCH_2NH_3^+$), 50.1 [1 H, t, J = 6 Hz, $ArCH(CH_2)NH_2^+$], 7.32 (4 H, br s, Ar*H*); MS, *m/e* 132 (100). No molecular ion was observed; $C_{10}H_{14}N_2$ requires *m/e* 162. Anal. ($C_{14}H_{18}N_2O_8$) C, H, N.

1-[(Isopropylamino)methyl]-1,2,3,4-tetrahydroisoquinoline (11) Dihydrochloride. 1-(Aminomethyl)-2-benzyl-1,2,3,4-tetrahydroisoquinoline (9; 3.1 g, 12 mmol) in a mixture of methanol (40 mL), acetone (10 mL), and concentrated hydrochloric acid (2.4 mL, 24 mmol) was treated with sodium cyanoborohydride (1.9 g, 30 mmol), as in the preparation of **4b**, to give 2-benzyl-1-[(isopropylamino)methyl]-1,2,3,4-tetrahydroisoquinoline (3.5 g, 97%): IR 3290 cm^{-1}; ^1H NMR δ 1.00 and 1.10 [3 H, d, J = 7 Hz, $CH(CH_3)_2$], 2.4 (br, 1 H, exchangeable, NH), 2.3–3.4 (7 H, m, $ArCH_2CH_2NH$, CH_2NH, and $CHNH$), 3.55–4.0 (1 H, m, $ArCHN$), 3.70 (2 H, s, $ArCH_2N$), 6.9–7.5 (9 H, m, Ar*H*). Of this, 3.4 g was hydrogenated over 5% palladium on charcoal (350 mg) in a mixture of 96% ethanol (80 mL) and concentrated hydrochloric acid (2.5 mL) to give, after recrystallization from ethanol, the **tetrahydroisoquinoline (11) dihydrochloride** (1.75 g, 55%): mp 230–238 °C dec; IR 3100–2300 cm^{-1}; ^1H NMR (D_2O; external Me_4Si) δ 1.37 [6 H, d, J = 7 Hz, $CH(CH_3)_2$], 2.9–3.35 (2 H, m, $ArCH_2CH_2$), 3.4–3.95 [5 H, m, $CH_2CH_2NH_2^+$ and $CHCH_2NH_2CH(CH_3)_2^+$], 5.08 [1 H, d of d, J_{AB} = 7.5 Hz, $J_{AB'}$ = 5 Hz, $ArCH(CH_2)NH_2^+$], 7.36 (4 H, s, Ar*H*). Anal. ($C_{13}H_{22}Cl_2N_2$) C, H, N. The free base gave IR 3260 cm^{-1}; ^1H NMR δ 1.05 [6 H, d, J = 6 Hz, $CH(CH_3)_2$], 1.88 (2 H, s, exchangeable, NH and NH), 2.5–3.2 (7 H, m, $ArCH_2CH_2NH$, CH_2NH, and $CHNH$), 3.97 (1 H, d of d, J_{AB} = 8 Hz $J_{AB'}$ = 5 Hz, $ArCHNH$), 7.04 (4 H, s, Ar*H*); ^1H NMR (D_2O; DSS) δ 1.07 (6 H, d, J = 7Hz), 2.5–3.2 (7 H, m), 4.02 (1 H, d of d, J_{AB} = 8 Hz, $J_{AB'}$ = 5.5 Hz), 7.16 (4 H, s), assignments as for $CDCl_3$ spectrum.

Equimolar amounts of the free base and the dihydrochloride salt were mixed together and recrystallized from ethanol-ether to give the monohydrochloride salt: mp 146–149 °C; IR 3280, 3100–2300 cm^{-1}; ^1H NMR (D_2O; external Me_4Si) δ 1.20 [6 H, d, J = 7 Hz, $CH(CH_3)_2$], 2.4–3.5 (7 H, m, $ArCH_2CH_2NH$, $CH_2NH_2^+$ and $CHNH_2^+$), 4.15 (1 H, d of d, J_{AB} = 8.5 Hz, $J_{AB'}$ = 5 Hz, $ArCHNH$), 7.05 (4 H, s, Ar*H*). The ^{13}C NMR spectra of the free base, mono-, and dihydrochloride salts are given in Table VII.

1-(Aminomethyl)-2-benzyl-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline (13, R = Me) Dihydrochloride Hemihydrate. A solution of potassium cyanide (2 g, 31 mmol) in water (20 mL) was added to a suspension of 2-benzyl-6,7-dimethoxy-3,4-dihydroisoquinolinium bromide (7.5 g, 21 mmol), and the mixture was extracted with ether to give 2-benzyl-1-cyano-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline (6.3 g, 98%): ^1H NMR δ 2.4–3.2, (4 H, m, $ArCH_2CH_2N$), 3.70 and 3.74 (3 H, s, OCH_3), 3.72 (2 H, s, $ArCH_2N$) 4.54 [1 H, s, $ArCHN(CN)$], 6.51 and 6.53 (1 H, s, Ar*H*), 7.1–7.5 (5 H, m, Ar*H*). A portion recrystallized from ether–petrol (bp 60–80 °C) had mp 107–108 °C; IR 2220 cm^{-1}. A solution of the nitrile (12, R = Me; 4.15 g, 13.5 mmol) in a mixture of dry ether (100 mL) and dry tetrahydrofuran (5 mL) was added to a cooled, stirred solution of aluminum hydride (79 mmol) in dry ether (150 mL), and the mixture was stirred at ambient temperature for 16 h to give 1-(aminomethyl)-2-benzyl-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline (13, R = Me; 3.9 g, 92%): IR 3350 cm^{-1}; ^1H NMR δ 1.7 (br, 2 H, exchangeable, NH_2), 2.3–3.3 (6 H, m, $ArCH_2CH_2N$ and $CHCH_2NH_2$), 3.51 [1 H, t, J = 6 Hz, $ArCH(CH_2)N$], 3.73 (2 H, s, NCH_2Ph), 3.78 (6 H, s, 2 OCH_3), 6.55 (2 H, s, Ar*H*), 7.1–7.4 (5 H, m, Ar*H*). The **dihydrochloride hemihydrate** had mp 206–208 °C (from 2-propanol–ether). Anal. ($C_{19}H_{28}Cl_2N_2O_2{\cdot}0.5H_2O$) C, H, N.

1-(Aminoethyl)-2-benzyl-6,7-bis(benzyloxy)-1,2,3,4-tetrahydroisoquinoline (13, R = CH_2Ph) Dihydrochloride. Phosphorus pentachloride (45 g, 216 mmol) was added portionwise to a cooled, stirred solution of *N*-[2-[3,4-bis(benzyloxy)phenyl]-ethyl]formamide (31 g, 86 mmol) in dry chloroform (200 mL), and the mixture was stirred overnight at ambient temperature. Dry ether (120 mL) was added, and the precipitate was filtered off, immediately added to water (150 mL), and stirred for 24 h. The resulting fine precipitate was filtered off, resuspended in water, basified with sodium hydroxide, and extracted with ethyl acetate to give 6,7-bis(benzyloxy)-3,4-dihydroisoquinoline (19.1 g, 65%). Benzyl bromide (13.5 mL, 113 mmol) was added to a solution of the dihydroisoquinoline (19 g, 55 mmol) in dry benzene (300 mL), and the mixture was stirred at ambient temperature for 64 h. The precipitate was filtered off and washed with ether to give the crude

(18) H. Boehme and R. Schweitzer, *Arch. Pharm.* (*Weinheim, Ger.*), **303**, 225 (1970).
(19) J. Z. Ginos, G. C. Cotzias, E. Tolosa, L. C. Tang, and A. LoMonte, *J. Med. Chem.*, **18**, 1194 (1975).

product (19.7 g), which was recrystallized from ethanol–ethyl acetate–ether to give the *N*-benzyldihydroisoquinolinium bromide (16.2 g, 57%): mp 165–166 °C; IR 1640 cm^{-1}; ^1H NMR δ 3.05 (2 H, t, J_{app} = 8 Hz, $ArCH_2CH_2$), 3.80 (2 H, t, J_{app} = 8 Hz, $CH_2CH_2N^+$), 5.07 and 5.18 (2 H, s, OCH_2Ph), 5.39 (2 H, s, NCH_2Ph^+), 6.90 and 7.78 (1 H, s, ArH), 7.0–7.7 (15 H, m, ArH), 10.31 (1 H, s, C_1 H).

A solution of potassium cyanide (4 g, 61 mmol) in water (50 mL) was added to a solution of 2-benzyl-6,7-bis(benzyloxy)-3,4-dihydroisoquinolinium bromide (16.2 g, 31 mmol) in methanol (100 mL), methanol was then evaporated off, and the aqueous residue was extracted with ethyl acetate to give 2-benzyl-1-cyano-6,7-bis(benzyloxy)-1,2,3,4-tetrahydroisoquinoline (14 g, 96%): IR 2220 cm^{-1}; ^1H NMR δ 2.4–3.1 (4 H, m), 3.78 (2 H, s), 4.50 (1 H, s), 4.98 and 5.04 (2 H, s), 6.64 (2 H, s), 7.0–7.5 (15 H, m), which was dissolved in a mixture of dry ether (200 mL) and dry tetrahydrofuran (50 mL), added to aluminum hydride (153 mmol) in dry ether (250 mL), and stirred overnight to give a mixture of products. The mixture was dissolved in ethanol and dripped into ethereal hydrogen chloride, and the precipitate was collected and recrystallized from acetone to give 2-benzyl-6,7-bis(benzyloxy)-1,2,3,4-tetrahydroisoquinoline hydrochloride (2.65 g, 18.5%): IR 2600–2200 cm^{-1}; ^1H NMR δ 2.85–3.50 (4 H, m, $ArCH_2CH_2NH^+$), 4.02 (2 H, br s, $ArCH_2NH^+$), 4.18 (2 H, s, NCH_2Ph^+), 5.00 and 5.04 (2 H, s, OCH_2Ph), 6.50 and 6.66 (1 H, s, ArH), 7.1–7.7 (15 H, m, ArH), 8.0–10.0 (1 H, exchangeable, NH^+). A portion recrystallized from ethanol had mp 183–186 °C. Anal. ($C_{30}H_{30}ClNO_2$) C, H, N. The free base gave δ 2.67 (4 H, br s, $ArCH_2CH_2N$), 3.46 (2 H, br s, $ArCH_2N$), 3.58 (2 H, s, NCH_2Ph), 5.00 and 5.02 (2 H, s, OCH_2Ph), 6.53 and 6.65 (1 H, s, ArH), 7.1–7.7 (15 H, m, ArH). The filtrate from the preceding crystallization was evaporated, and the residue was purified from 2-propanol–acetone to give 1-(aminomethyl)-2-benzyl-6,7-bis(benzyloxy)-1,2,3,4-tetrahydroisoquinoline (**13**, R = CH_2Ph) dihydrochloride (8 g, 49%): mp 177–181 °C; IR 3100–2200 cm^{-1}. Anal. ($C_{31}H_{34}Cl_2N_2O_2$) C, H, N. The free base gave IR 3350 cm^{-1}; ^1H NMR δ 1.4 (br, 2 H, exchangeable, NH_2), 2.45–3.2 (6 H, m, $ArCH_2CH_2N$ and $CHCH_2NH_2$), 3.3–3.6 [1 H, m, $ArCH(CH_2)N$], 3.70 (2 H, s, NCH_2Ph), 5.06 (4 H, s, 2 OCH_2Ph), 6.58 and 6.63 (1 H, s, ArH), 7.0–7.7 (15 H, m, ArH).

1-(Aminomethyl)-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (4a) Dihydrochloride Hemihydrate. 1-(Aminomethyl)-2-benzyl-6,7-bis(benzyloxy)-1,2,3,4-tetrahydroisoquinoline (2.2 g, 5 mmol) in a mixture of methanol (150 mL) and concentrated hydrochloric acid (2 mL) was hydrogenated over 5% palladium on charcoal (0.6 g), the mixture was filtered through Kieselguhr, the filtrate was evaporated, and the residue was recrystallized from methanol–acetone–ether to give the **tetrahydroisoquinoline (4a) dihydrochloride hemihydrate** (0.93 g, 71%): mp 135–143 °C; IR 3400, 3300–2300 cm^{-1}; ^1H NMR (D_2O; DSS) δ 2.85–3.25 (2 H, m, $ArCH_2CH_2$), 3.45–3.90 (4 H, m, $CH_2CH_2NH_2^+$ and $CHCH_2NH_3^+$), 4.95 [1 H, t, J = 6 Hz, $ArCH(CH_2)NH_2^+$], 6.79 and 6.84 (1 H, s, ArH). Anal. ($C_{10}H_{16}Cl_2N_2O_2$·0.5H_2O) C, H, N.

6,7-Dihydroxy-1-[(isopropylamino)methyl]-1,2,3,4-tetrahydroisoquinoline (4b) Dihydrochloride. Sodium cyanoborohydride (0.85 g, 13.5 mmol) was added portionwise to a cooled, stirred solution of 1-(aminomethyl)-2-benzyl-6,7-bis(benzyloxy)-1,2,3,4-tetrahydroisoquinoline (2.5 g, 5.4 mmol) in a mixture of methanol (30 mL), acetone (5 mL), and concentrated hydrochloric acid (1.1 mL, 11 mmol), and the mixture was stirred at ambient temperature for 16 h. The mixture was acidified with dilute hydrochloric acid, organic solvents were evaporated off, and the residue was basified with sodium bicarbonate and extracted with ethyl acetate to give 2-benzyl-6,7-bis(benzyloxy)-1-[(isopropylamino)methyl]-1,2,3,4-tetrahydroisoquinoline (2.5 g, 92%): IR 3275 cm^{-1}; ^1H NMR δ 0.98 and 1.03 [6 H, d, J = 7 Hz, $CH(CH_3)_2$], 2.3–3.3 (8 H, becoming 7 H with D_2O, m, $ArCH_2CH_2N$, CH_2N, and CHN), 3.4–3.8 (1 H, m, $ArCHN$), 3.69 (2 H, s, $ArCH_2N$), 5.06 (4 H, s, $ArCH_2O$), 6.63 and 6.66 (1 H, s, ArH), 7.1–7.6 (15 H, m, ArH). The dihydrochloride salt, which had mp 113–123 °C (from 2-propanol–ether), IR 3100–2200 cm^{-1} (2.1 g, 4 mmol), in methanol (150 mL) was hydrogenated over 5% palladium on charcoal (0.5 g), the mixture was filtered through Kieselguhr, the filtrate was evaporated to dryness, and the residue was purified from ethanol to give **tetrahydroisoquinoline (4b) dihydrochloride** (0.7 g, 64%): mp 241–243 °C dec; IR 3410, 3270, 3100–2200 cm^{-1}; ^1H NMR (D_2O, DSS) δ 1.43 [6 H, d, J = 7 Hz, $CH(CH_3)_2$], 2.85–3.25 (2 H, m, $ArCH_2CH_2$), 3.35–3.90 [5 H, m, $CH_2CH_2NH_2^+$ and $CHCH_2NH_2CH(CH_3)_2^+$], 4.75–5.15 [1 H, m, $ArCH(CH_2)NH_2^+$], 6.80 and 6.87 (1 H, s, ArH); MS *m/e* 236 (M^+, 5), 177 (37), 164 (46), 163 (59), 162 (54), 149 (41), 72 (100). A portion recrystallized from aqueous methanol–ether had mp 252–254 °C dec (lit.[5] mp 268–270 °C dec). Anal. ($C_{13}H_{22}Cl_2N_2O_2$) C, H, N.

1-[(*tert*-Butylamino)methyl]-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (4c) Dihydrochloride. A mixture of bromoacetaldehyde diethyl acetal (20.3 g, 0.103 mol), *tert*-butylamine (50 mL, 0.47 mol), and dry benzene (50 mL) was boiled under reflux for 48 h, cooled, and evaporated almost to dryness. The residue was dissolved in ether and washed with dilute sodium hydroxide, and the organic layer was evaporated to dryness. The residue was distilled (90 °C at 1.5 mmHg) to give *N*-(*tert*-butylamino)acetaldehyde diethyl acetal (15.6 g, 80%): decomposes at 160 °C at 758 mmHg; IR 3320 cm^{-1}; ^1H NMR δ 1.08 (9 H, s), 1.20 (6 H, t, J = 7 Hz), 0.9–1.4 (1 H, exchangeable), 2.66 (2 H, d, J = 6 Hz), 3.23–3.90 (4 H, m), 4.54 (1 H, t, J = 6 Hz). A mixture of the acetal (3 g, 15.9 mmol), dopamine hydrochloride (2 g, 10.6 mmol), 1-butanol (40 mL), concentrated hydrochloric acid (2.4 mL), and water (5 mL) was boiled under reflux in a nitrogen atmosphere for 4 h. The progress of the reaction was followed by TLC on silica eluting with ethyl acetate–acetic acid–water (15:15:10, v/v) and visualizing with 1% ferric chloride solution. The mixture was evaporated to dryness, the residue was dissolved in ethanol and filtered, and the filtrate was dripped into dry ether. The precipitate was collected and recrystallized from 2-propanol–acetone–isopropyl ether to give the **tetrahydroisoquinoline (4c) dihydrochloride** (1.1 g, 32%): mp 239–242 °C dec; IR 3330, 3075–2350 cm^{-1}; ^1H NMR (D_2O; DSS) δ 1.46 [9 H, s, $C(CH_3)_3$], 2.85–3.25 (2 H, m, $ArCH_2CH_2$), 3.4–3.8 (4 H, m, $CH_2CH_2NH_2^+$ and $CHCH_2NH_2^+$), 4.7–5.1 [1 H, m, $ArCH(CH_2)NH_2^+$], 6.78 and 6.85 (1 H, s, ArH); MS, *m/e* 250 (M^+-, 13), 164 (68), 163 (72), 162 (70), 86 (100). Anal. ($C_{14}H_{24}Cl_2N_2O_2$) C, H, N.

pK_a Determination of 1-(Aminomethyl)- and 1-[(Isopropylamino)methyl]-1,2,3,4-tetrahydroisoquinolines. The pH of a solution of the test substance (1.5–2 mmol) in distilled water (50–100 mL) titrated with N/20 hydrochloric acid, delivered at a rate of 2.4 mL/min by a Watson–Marlow flow Inducer Type 22, was continually monitored by a Pye-Unicam 401 combined electrode linked through a Pye-Unicam PW9418 pH meter to a chart recorder. From the graph of pH vs. volume of titrant added, the pH at half neutralization was taken as a measure of pK_a. Values obtained were as follows: 1-(aminomethyl)-1,2,3,4-tetrahydroisoquinoline (**10**), 5.7 and 8.9; *N*-isopropyl analogue **11**, 5.4 and 9.2. The bases **10** and **11** were generated from their dioxalate and hydrochloride salts, respectively, immediately before use.

The experimentally determined values were obtained at 20 °C. It is probable that values at 37 °C would be about 0.3 pK_a units lower.[20] It is also probable that the introduction of 6,7-dihydroxy groups would alter the amine pK_a values slightly, since 2-phenylethylamine is reported[21] to have pK_a of 9.88 at 25 °C, whereas dopamine is reported[22] to have pK_a of 10.63 at 20 °C. These considerations do not alter the main conclusion that the diamines under consideration would be substantially monoprotonated under physiological conditions.

Pharmacological Methods. Guinea Pig, Isolated Preparations. Guinea pigs (300–400 g) of either sex were killed by cervical dislocation. Right atrial and tracheal preparations were removed and mounted in 10-mL tissue baths containing Krebs–Henseleit solution of the following composition (mmol/L): NaCl, 118; $NaHCO_3$, 24.9; KCl, 4.7; $MgSO_4$·$7H_2O$, 1.2; $CaCl_2$, 1.9; KH_2PO_4, 1.2; glucose, 11.1 at 37 °C. It was bubbled with 5% CO_2 in O_2. The bathing solution contained ethylenediaminetetraacetic acid (EDTA, 67 μmol/L) and ascorbic acid (10 μmol/L) to reduce oxidation of catecholamines. In all experiments a 30- to 60-min

(20) A. Albert and E. P. Serjeant, "Ionization Constants of Acids and Bases. A Laboratory Manual", Methuen, London, 1962.
(21) T. Kappe and M. D. Armstrong, *J. Med. Chem.*, 8, 368 (1965).
(22) B. R. Martin, *J. Phys. Chem.*, 75, 2657 (1971).

equilibration period was allowed before addition of drugs to the tissue bath. In some experiments, guinea pigs were injected intraperitoneally with reserpine (Serpasil, Ciba Chemical Co.) in a dose of 5 mg/kg. Tissues were removed for study 18–24 h later. These preparations were tested with tyramine at a concentration of 17 μmol/L before commencing further experiment.

Right Atria. A tension of 0.02 N (2 g wt) was applied to the atrium. Contractions were recorded with a Grass force-displacement transducer (FT03), and the rate was recorded with a Grass tachograph (7P44B1) on a polygraph (790).

To obtain agonist activity, two cumulative concentration–response curves to isoproterenol and a single cumulative concentration–response curve for the test compound were established with 30-min intervals. The bath was washed three times at the end of each cumulative determination. In control experiments, three successive concentration–response curves for isoproterenol were established. Slight desensitization occurred: between curves two and three the factor was 1.2. Therefore, in experiments with test compounds, only the second isoprenaline dose–response relationship was used for potency determination, and appropriate correction was made to the $E_{max(50)}$ value for the test compound.

For determination of antagonistic activity, a concentration–effect curve for isoproterenol was established before and 15 min after the drug under test was added to the tissue bath. After washing, the process was repeated with higher concentration of the drug under test. pA_2 values were determined by the method of Arunlakshana and Schild.[23]

Trachea. Tracheae were removed and placed in Krebs–Henseleit solution bubbled with CO_2 in O_2 at room temperature and then cleared of connective tissue. An incision was made longitudinally in the cartilaginous band opposite to the smooth-muscle band. Transverse cuts through approximately three-quarters of the width of the preparation at two to three ring intervals were then made alternately from each side of the trachea. A near vertical alignment of the smooth-muscle fibers along the axis through which tension was measured was thus obtained. A resting tension of 0.02 N (2 g wt) was applied to the preparation in the tissue bath, and tension was recorded as for atria.

Carbachol (10 μmol/L) was used to induce a submaximal increase in tone in the preparations. Two cumulative concentration–response curves for isoproterenol and then a single curve for the test drug were established with 30-min intervals. The bath was washed three times after each determination. Only the second curve for isoproterenol was used in potency determinations, and, as with atria, a desensitization factor (1.3) was applied to the results for the test compound. The maximum response to all drugs under test was less than that of isoproterenol, a maximum response to which was reestablished during the period of maximum response to the drug under test. pD_2 values were obtained as with atria.

Antagonistic action was established with a procedure the same as that used with atria, except that the preparations were continuously exposed to carbachol (1 μmol/L).

Anesthetized Cats. Cats of either sex and weighing 2.3–3.6 kg were anesthetized by intraperitoneal injection of a mixture of chloralose (80 mg/kg) and sodium phenobarbitone (6 mg/kg). The trachea was cannulated, but the cat was allowed to breathe spontaneously. Drugs were injected into a brachial vein. Blood pressure was recorded from a cannulated common carotid artery by using a Druck pressure transducer (PDGR75), and heart rate was recorded by using a Grass Tachograph triggered by the arterial pulse.

After constant submaximal responses to a single dose of isoproterenol, concurrent cumulative dose–response curves for effects on the heart rate and blood pressure were established. Effects of isoproterenol were first determined, followed by responses to other drugs. In each experiment, one cumulative dose–response relationship was determined. Dose–response curves were plotted in terms of percentage of the maximum responses produced by isoproterenol.

Drugs. Drugs used were isoproterenol hydrochloride (Sterling Pharmaceuticals), tetrahydropapaveroline hydrobromide (Wellcome Research Laboratories), propranolol hydrochloride (Imperial Chemical Industries), carbachol (injection BP, Abbott Laboratories), reserpine (Serpasil, Ciba-Geigy), tyramine hydrochloride (Koch-Light), α-chloralose (British Drug Houses, and pentobarbitone sodium (Nembutal sodium, Abbott Laboratories). All drugs solutions, except reserpine, carbachol, and pentobarbitone sodium, were weighed and diluted to the appropriate concentrations with Krebs–Henseleit solution for in vitro preparations or 0.9%, w/v, NaCl solution for anesthetized cat preparations at the beginning of each experiment. The commercial carbachol, reserpine, and pentobarbitone sodium preparations were used undiluted. All working solutions were kept on ice during the experimental period.

Acknowledgment. D.B. was supported by a S.R.C. studentship. We thank Dr. C. W. Thornber for his interest and for securing preliminary pharmacological testing.

Registry No. **4a**, 84500-62-9; **4a**·2HCl, 60085-38-3; **4b**, 84500-63-0; **4b**·2HCl, 60085-35-0; **4c**, 84500-64-1; **4c**·2HCl, 84500-65-2; **5**, 84500-66-3; **6** (R = H), 84500-67-4; **6**·HCl (R = H), 27002-40-0; **6** (R = CH_2Ph), 37039-47-7; **8** dioxolate, 84500-69-6; **9**, 40615-06-3; **10**, 84500-70-9; **10** dioxolate, 84500-71-0; **11**, 84500-72-1; **11**·HCl, 84500-73-2; **11**·2HCl, 84500-74-3; **12** (R = Me), 73154-44-6; **12** (R = CH_2Ph), 84500-75-4; **13** (R = Me), 84500-76-5; **13**·HCl (R = Me), 84500-77-6; **13** (R = CH_2Ph), 84500-78-7; **13**·HCl (R = CH_2Ph)·2HCl, 84500-79-8; **14**, 84500-80-1; **14**·2HCl, 84500-81-2; benzyl bromide, 100-39-0; 6,7-dimethoxy-3,4-dihydroisoquinoline, 3382-18-1; *N*-benzyl-6,7-dimethoxy-3,4-dihydroisoquinolinium bromide, 5096-82-2; potassium cyanide, 151-50-8; 3,4-dihydroisoquinoline, 3230-65-7; lithium aluminum hydride, 16853-85-3; sodium bis(2-methoxyethoxy)aluminum hydride, 22722-98-1; aluminum hydride, 7784-21-6; 2-benzyl-3,4-dihydroisoquinolinium bromide, 84500-82-3; acetone, 67-64-1; 2-benzyl-1-[(isopropylamino)methyl]-1,2,3,4-tetrahydroisoquinoline, 84500-83-4; *N*-[2-[3,4-bis(benzyloxy)phenyl]ethyl]formamide, 84500-84-5; 6,7-bis(benzyloxy)-3,4-dihydroisoquinoline, 84500-85-6; *N*-benzyl-6,7-bis(benzyloxy)-3,4-dihydroisoquinolinium bromide, 84500-86-7; 2-benzyl-6,7-bis(benzyloxy)-1,2,3,4-tetrahydroisoquinoline hydrochloride, 84500-87-8; 2-benzyl-6,7-bis(benzyloxy)-1,2,3,4-tetrahydroisoquinoline, 84500-88-9; bromoacetaldehyde diethyl acetal, 2032-35-1; *tert*-butylamine, 75-64-9; *N*-(*tert*-butylamino)acetaldehyde diethyl acetal, 84500-89-0; dopamine hydrochloride, 62-31-7.

(23) O. Arunlakshana and H. O. Schild, *Br. J. Pharmacol.*, 14, 48 (1959).

(24) P. Cerutti and H. Schmidt, *Helv. Chim. Acta*, 47, 203 (1964).

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Synthesis and Analgesic Properties of Two Leucine-enkephalin Analogues Containing a Conformationally Restrained N-Terminal Tyrosine Residue

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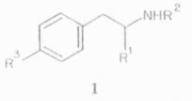
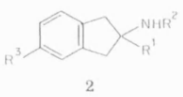
Two analogues of Leu-enkephalin, in which the terminal tyrosine-1 residue has been replaced by a conformationally restrained tyrosine analogue, have been synthesized by classical solution methods, and their opiate agonist potencies on electrically stimulated guinea pig ileum and mouse vas deferens preparations were determined in comparison with Leu-enkephalin. The restriction in the degree of freedom of the tyrosine moiety in [(2-amino-6-hydroxy-2-tetralinyl)carbonyl]glycylglycylphenylalanylleucine methyl ester (**3e**) leads to a 7 to 8 times higher agonist activity at the μ-receptor subtype in guinea pig ileum when compared to Leu-enkephalin, and an almost 30-fold decrease in potency, vs. Leu-enkephalin, on mouse vas deferens preparation. [(2-Amino-5-hydroxy-2-indanyl)carbonyl]-glycylglycylphenylalanylleucine methyl ester (**2e**) was inactive in the above tests. These results demonstrate the differential effect of restricting conformational flexibility on receptor recognition. Neither analogue had any analgesic properties when evaluated by the hot-plate test in mice after sc and icv administration.

The role of enkephalins as natural endogenous ligands for the opiate receptor in the central nervous system is now well established.[1,2] A large number of structural analogues of both leucine- and methionine-enkephalins (**1g** and **1f**, respectively) have been synthesized over the past 5 years,



1



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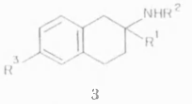

3

4

a, R^1 = COOH; R^2 = H; R^3 = OH
b, R^1 = COOH; R^2 = H; R^3 = $OCH_2C_6H_5$
c, R^1 = COOH; R^2 = $COOCH_2C_6H_5$; R^3 = $OCH_2C_6H_5$
d, R^1 = CO-Gly-Gly-L-Phe-L-Leu-OCH_3; R^2 = $COOCH_2C_6H_5$; R^3 = $OCH_2C_6H_5$
e, R^1 = CO-Gly-Gly-L-Phe-L-Leu-OCH_3; R^2 = H; R^3 = OH
f, R^1 = CO-Gly-Gly-L-Phe-L-Leu-OH; R^2 = H; R^3 = OH
g, R^1 = CO-Gly-Gly-L-Phe-L-Met-OH; R^2 = H; R^3 = OH
h, R^1 = CO-D-Ala-Gly-L-Phe-L-Leu-OCH_3; R^2 = H; R^3 = OH

which have been aimed at affording active compounds having greater stability toward metabolizing enzymes and/or to facilitate entry into the central nervous system after oral or peripheral administration.[3] Such structural modifications include shortening or lengthening of the pentapeptide chain, substitution of individual amino acids by other amino acids, and chemical modification of individual amino acids, particularly terminal amino acids. Numerous conformational studies[4] have been carried out that propose particular "active" conformations for enkephalins at the analgesic receptor, and the structural analogy between enkephalins and morphine[5] has been discussed. This analogy emphasizes the close correspondence between the tyramine moiety in the morphine molecule and the phenolic ring and amino group of the terminal tyrosine residue in the enkephalins. Since morphine is a rigid molecule, this infers that the flexible tyrosine moiety in the enkephalins interacts at the analgesic receptor in a specific conformation that is presumably stereochemically compatible with the tyramine unit in the rigid morphine molecule.

Leucine-enkephalin (Leu-enkephalin, **1f**) has been crystallized and its structure determined.[6] X-ray crystallographic data suggest that the orientation of the tyrosine side chain is not unique, the tyrosine aromatic ring experiencing disorder of either a static or dynamic nature. A solid-state NMR investigation of the Leu-enkephalin structure[7] has recently shown that in the polycrystalline state, the aromatic tyrosine ring is undergoing 180° flipping about the C^β–C^γ axis at a rate of approximately 5×10^4 s^{-1} at room temperature.

In order to explore the effect of a restriction in the degree of conformational freedom of the tyrosine moiety on the biological properties of Leu-enkephalin analogues, we have synthesized two analogues, **2e** and **3e**, of Leu-enkephalin in which the terminal tyrosine unit has been

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(1) J. A. H. Lord, A. A. Waterfield, J. Hughes, and H. W. Kosterlitz, "Opiates and Endogeneous Opioid Peptides", H. W. Kosterlitz, Ed., North-Holland Publishing Co., Amsterdam, 1976.
(2) S. H. Synder, *Sci. Am.*, **236**, 44 (1977).
(3) For a recent review, see J. Morley, *Annu. Rev. Pharmacol. Toxicol.*, **20**, 81 (1980), and references cited therein.
(4) S. Combrisson, B. P. Roques, and R. Oberlin, *Tetrahedron Lett.*, 3455 (1976); H. E. Bleich, J. D. Cutnell, A. R. Day, R. J. Freer, J. A. Glasel, and J. F. McKelvy, *Proc. Natl. Acad. Sci. U.S.A.*, **73**, 2589 (1976); B. P. Roques, C. Garby-Jaurequiberry, and R. Oberlin, *Nature (London)*, **262**, 778, (1976); C. R. Jones, W. A. Gibbons and V. Garskey, *ibid.*, **262**, 779; (1976); E. R. Stimson, Y. C. Meinwald, and H. A. Scheraga, *Biochemistry*, **18**, 1661 (1979) and references cited therein; J. DiMaio, P. W. Schiller, and B. Belleau, "Peptides, Structure and Biological Function", Proceedings of the American Peptide Symposium, 6th, Georgetown University, Washington, DC, June 17–22, 1978 E. Gross and J. Meienhofer, Eds., Pierce Chemical Co., Rockford, IL, 1979; M. C. Summers and R. J. Hayes, *FEBS Lett.*, **113**, 99–101 (1980); D. Hudson, R. Sharpe, and M. Szelke, *Int. J. Protein Res.*, **15**, 122 (1980); J. S. Shaw and M. J. Turnbull, *Eur. J. Pharmacol.*, **49**, 313, (1978).
(5) A. S. Horn and J. R. Rodgers, *Nature (London)*, **260**, 795 (1976); B. E. Maryanoff and M. J. Zelesko, *J. Pharm. Sci.*, **67**, 590 (1978); R. H. B. Galt, *J. Pharm. Pharmacol.*, **29**, 711 (1977); B. P. Roques, G. Gacel, M.-C. Fournie-Zaluski, B. Senault and J.-M. Lecomte, *Eur. J. Pharmacol.*, **60**, 109 (1979). J. DiMaio, and P. W. Schiller, *Proc. Natl. Acad. Sci. U.S.A.*, **77**, 7162 (1980).
(6) G. D. Smith and J. F. Griffin, *Science*, **199**, 1214 (1978); T. L. Blundell, L. Hearn, I. J. Tickle, R. A. Palmer, B. A. Morgan, G. D. Smith, and J. F. Griffin, *ibid.*, **205**, 220 (1979).
(7) D. M. Rice, R. J. Wittebort, R. G. Griffin, E. Meirovitch, E. R. Stimson, Y. C. Meinwald, J. H. Freed and H. A. Scheraga, *J. Am. Chem. Soc.*, **103**, 7707 (1981).

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Table I. Opioid Activities of Leucine-enkephalin Analogues

	guinea pig ileum[a]		mouse vas deferens[a]	
compd	rel potency (morphine = 1)	ID_{50}, nM (95% CL)	rel potency (morphine = 1)	ID_{50}, nM (95% CL)
2e	0.03		0.02[b]	
3e	1.38	65.3 (32.1-110.2)	2.91[c]	333.4 (261.7-460.1)
1g[d]	1.06	90.1 (31.3-190.5)	55.32	17.3 (6.8-52.4)
1f[e]	0.11	504.2 (297.2-881.2)	89.38	12.1 (5.6-20.9)
1e[f]	no inhibn		8.31	201.2 (142.1-243.1)

[a] Data are from four determinations for each compound. [b] 37% naloxone reversible at 27 μg/mL. [c] 100% naloxone reversible at 27 μg/mL. [d] Methionine-enkephalin. [e] Leucine-enkephalin. [f] Leucine-enkephalin methyl ester.

modified by incorporating it into different ring systems and in which the terminal leucine-5 residue has been esterified to aid passage into the CNS. These analogues have been evaluated for analgesic activity on isolated guinea pig ileum myenteric plexus muscle and mouse vas deferens tissue and by the hot-plate test in mice after icv administration.

Chemistry. 2-Amino-5-hydroxy-2-indancarboxylic acid (2a) and 2-amino-6-hydroxy-tetralincarboxylic acid (3a) were prepared by literature methods[8,9] and obtained as racemates. The tetrapeptide H-Gly-Gly-Phe-Leu-OCH_3 (4) was synthesized by classical solution methods.[12] Preparation of the pentapeptides 2e and 3e were carried out as follows. The racemic tyrosine analogues 2a and 3a were N protected with carbobenzoxy chloride in base, O protected with benzyl bromide, and then coupled with tetrapeptide 4 by using *N,N'*-dicyclohexylcarbodiimide and 1-hydroxybenzotriazole. Hydrogenolysis of the fully protected pentapeptides 2d and 3d and purification of the resulting deprotected products by gradient buffer elution from carboxymethylcellulose at pH 5.1 afforded the pentapeptide methyl esters 2e and 3e. Both pentapeptides were obtained as a mixture of their respective diastereoisomers.

Pharmacology. The opiate agonist potential of the pentapeptide methyl esters 2e and 3e were carried out on electrically stimulated guinea pig ileum and in mouse vas deferens preparations and are summarized in Table I. In the guinea pig ileum, the analgesic actions of enkephalins and opiates are mediated by their interaction with the μ receptor, whereas in mouse vas deferens, a different type of receptor, the δ receptor, predominates.[10] This latter receptor type interacts more strongly with opioid peptides than with the classical opiates.

Interestingly, a significant difference in potency was observed between 2e and 3e on both guinea pig ileum and mouse vas deferens preparations. The restriction in the degrees of freedom of the tyrosine moiety in 3e leads to a 7 to 8 times increase in agonist activity at the μ-receptor subtype as compared to Leu-enkephalin, coupled with an almost 30-fold decreased potency, vs. Leu-enkephalin, on the mouse vas deferens preparation. Neither of the two Leu-enkephalin analogues were active as analgesics when examined by the hot-plate test in mice after sc and icv injection.

The results from the isolated tissue experiments demonstrate the differential effect of restricted conformation on receptor recognition and indicate that opiate activity in Leu-enkephalin derivatives can be retained by replacement of the terminal tyrosine unit with a less flexible structural analogue. The difference in potency of almost two orders of magnitude between the indan derivative 2e and the tetralin derivative 3e is worthy of note. The drastic decrease in potency of the indan derivative 2e compared to 3e suggests a preferred conformation for the tyrosine moiety in Leu-enkephalin at the μ receptor. This finding warrants greater scrutiny. The greater potency of the Leu-enkephalin analogue 3e at the μ receptor as compared to the δ receptor is in accord with other studies, which indicate that greater structural flexibility is required in analgesic molecules for δ-receptor recognition than for μ-receptor binding. Shaw and Turnbull[4] have reported that the pentapeptide 3h is about 7 times more potent as an agonist on the guinea pig ileum than on mouse vas deferens preparations. Thus, 3e appears to resemble morphine, rather than Leu-enkephalin, in its receptor characteristics.

While the lack of in vivo activity of 3e is unlikely to be due to inadequate CNS penetration, metabolic factors may play a significant role in this respect, since it is possible that 3e could be a substrate for amino peptidase and/or enkephalinase enzymes.

Experimental Section

Melting points were determined on a Reichert hot-stage microscope and are uncorrected. Microanalyses were performed by the Micro-analytical Laboratory, Department of Chemistry, University of Manchester. Analytical results obtained for all compounds were within ±0.4% of the theoretical value unless otherwise stated. ^1H NMR sepctra were recorded on a Varian HR 220 or a Varian SC 300 spectrometer and are quoted on the δ scale. IR spectra were recorded as KCl disks, Nujol mulls, or liquid films on a Perkin-Elmer 237 spectrophotometer. Mass spectra were determined on an A.E.I. MS9 spectrometer operating at a probe temperature of 250 °C. Thin-layer chromatographic separations were carried out on 0.25-mm Polygram silica gel UV_{254}. 2-Amino-5-hydroxy-2-indancarboxylic acid[8] and 2-amino-6-hydroxy-2-tetralincarboxylic acid[9] were prepared by literature procedures. Amino acids and peptides with free amino functions were visualized by spraying with 1% ninhydrin in ethanol. N-Protected amino acids and peptides were visualized with 5% potassium dichromate in concentrated H_2SO_4, and amino acids and peptides possessing a phenolic hydroxy function were visualized with Pauli's reagent.[11] Other compounds were visualized in iodine vapor. Hydrogenations were carried out on a Gallenkampf hydrogenator operating at atmospheric pressure and room temperature.

Ion-exchange chromatographic separations of N-deprotected pentapeptides were carried out on Whatman CMC 52 carboxymethylcellulose, prewashed with 0.5 N aqueous sulfuric acid,

(8) R. M. Render, B. H. Butcher, D. A. Buxton, and D. J. Howells, *J. Med. Chem.*, 14, 892 (1971).
(9) S. N. Rastogi, J. S. Bindra, and N. Anand, *Indian J. Chem.*, 9, 1175 (1971).
(10) J. A. H. Lord, A. A. Waterfield, J. Hughes, and H. W. Kosterlitz, *Nature (London)*, 267, K.-J. Chang and P. Cuatrecasas, *J. Biol. Chem.*, 254, 2610 (1979); B. K. Handa, A. C. Land, J. A. Lord, B. A. Morgan, M. J. Rance, and C. F. Smith, *Eur. J. Pharmacol.*, 70, 531 (1981); Y. Kiso, T. Miyazaki, T. Akita, and H. Nakamura, *ibid.*, 71, 347 (1981); G. Gacel, M.-C. Fournie-Zaluski, and B. P. Roques, *FEBS Lett.*, 118, 245 (1980).
(11) E. G. Clark, "Isolation and Identification of Drugs in Pharmaceutical Body Fluids and Post Mortem Material", Pharmaceutical Press, London, 1969, p 803.
(12) E. Vilkas, M. Vilkas, and J. Sainton, *Int. J. Pept. Protein Res.*, 15, 29 (1980).

followed by 0.2 N aqueous ammonium acetate buffer adjusted to pH 5.1 with 0.880 specific gravity ammonia solution or glacial acetic acid and measured with a Pye-Unicam PW 9418 pH meter fitted with a Pye-Unicam 401 combined electrode. Ion-exchange columns were run under slight pressure with a peristaltic pump set for an effluent output of 100 mL/h. Column effluents were monitored by UV spectrophotometry at 280 nm with a Cecil CE 202 spectrophotometer fitted with a 10-mm path-length flow-through cell. Columns were eluted with 0.005–0.5 N gradient ammonium acetate buffer solutions at pH 5.1, and fractions were collected on an automatic fraction collector.

Amino acid analyses of peptides were performed on a Beckmann SPINCO Model 123 amino acid analyzer using ninhydrin as visualizer. The peptides were hydrolyzed in 6 N HCl at 110 °C for 24 h in a sealed, evacuated tube.

2-Amino-5-(benzyloxy)-2-indancarboxylic Acid (2b) and 2-Amino-6-(benzyloxy)-2-tetralincarboxylic Acid (3b). As a general method, the appropriately substituted 2-amino-2-carboxylic acid (0.039 mol) in 2 N NaOH solution (48 mL) was treated with copper sulfate ($CuSO_4 \cdot 5H_2O$, 5.92 g, 0.024 mol) in water (24 mL). The mixture was mechanically stirred at 20–25 °C for 30 min, and methanol (140 mL) and then benzyl bromide (5.90 mL, 0.041 mol) were added. Vigorous stirring was maintained at 22–25 °C for 15 h. The resulting blue copper complex was collected and washed with water (3 × 25 mL), methanol (2 × 25 mL), and diethyl ether (2 × 25 mL), and the air-dried product (11.4 g) was stirred for 30 min at 50–60 °C with concentrated HCl (10 mL) and water (100 mL). While the solution was cooling, a brown colloidal gel formed, which was difficult to filter. This gel was triturated with 2 N hydrochloric acid (100 mL) and centrifuged at 48000 rpm for 10 min. The green supernatant was decanted, and the gel was again stirred at 50–60 °C for 30 min with concentrated hydrochloric acid (10 mL) and water (100 mL). The resulting supernatant was decanted, and the residue was air-dried and crystallized from glacial acetic acid to afford the appropriately substituted 2-amino-2-carboxylic acid.

2-Amino-5-(benzyloxy)-2-indancarboxylic acid hydrochloride (2b) was prepared as described above from **2a** as white crystals (5.0 g, 40%): mp 220–225 °C; IR (Nujol) 3350, 3145, 3090, 3000, 1728, 1612, 1588 cm^{-1}; NMR (Me_2SO-d_6, 60 MHz) 3.35 (m, 4 H, C-1 and C-3 protons), 5.03 (s, 2 H, benzyl CH_2), 6.68–7.56 (m, 8 H, aromatic protons), 8.95 (br s, 4 H, replaceable with D_2O, NH_3 and COOH). Anal. ($C_{17}H_{17}NO_3$·HCl) C, H, N.

2-Amino-6-(benzyloxy)-2-tetralincarboxylic acid hydrochloride (3b) was prepared as described above from **3a** as white crystals (5.6 g, 43%): mp 212–215 °C; IR (KCl) 3370, 3030, 2890, 2650, 2550, 1720, 1605, 1573 cm^{-1}; NMR (TFA, 60 MHz) 2.55 (m, 2 H, C-3 protons), 3.07 (m, 2 H, C-4 protons), 3.42 (m, 2 H, C-1 protons), 5.19 (s, 2 H, benzylic CH_2), 6.70–7.65 (m, 8 H, aromatic protons). Anal. ($C_{18}H_{19}NO_3$·HCl) C, H, N.

Preparation of *N*-Benzyloxycarbonyl-Protected Amino Acids. As a general method, the appropriate amino acid (0.011 mmol) suspended in 2 N NaOH solution (1000 mL) was heated on a steam bath to dissolve the amino acid and then cooled in an ice bath to 0–5 °C, which caused the sodium salt of the amino acid to precipitate out. Carbobenzyloxy chloride (2.11 g, 0.012 mol) was added dropwise to the cooled, stirred mixture, over 40 min. The ice bath was then removed, and the mixture was stirred at room temperature for 30 h. The reaction mixture was cooled to 0–5 °C, and a further 2.11 g (0.012 mol) of carbobenzyloxy chloride was added dropwise. After a total reaction time of 60 h, the mixture was carefully adjusted to pH 1 with 5 N HCl and extracted with diethyl ether (2 × 100 mL). The combined organic extracts were dried, filtered, and evaporated to dryness, and the residue was crystallized from diethyl ether/petroleum ether (bp 30–40 °C) to afford the appropriate *N*-benzyloxycarbonyl-protected amino acid.

5-(Benzyloxy)-2-[(benzyloxy)carbamido]-2-indancarboxylic acid (2c) was prepared as described above from **2b** in 41% yield: mp 115.5–121 °C; IR (KCl) 3320, 2995, 2970, 2870, 1738, 1705, 1650, 1604, 1580, 1534 cm^{-1}; NMR ($CDCl_3$, 60 MHz) 3.37 (m, 4 H, C-1 and C-3 protons), 4.96 (s, 2 H, benzyl CH_2), 5.03 (s, 2 H, benzloxy CH_2), 6.60–7.95 (m, 14 H, reducing to 13 H on addition of D_2O, NH and aromatic protons), 9.78 (br s, 1 H, replaceable with D_2O, COOH); mass spectrum, *m/e* 417 (M^+). Anal. ($C_{25}H_{23}NO_5$) C, H, N.

6-(Benzyloxy)-2-[(benzyloxy)carbamido]-2-tetralincarboxylic acid (3c) was prepared as described above from **3b** in 52% yield: mp 150–156 °C; IR (KCl) 3380, 3255, 3050, 3020, 2945, 2915, 2840, 1718, 1710, 1685, 1605, 1545, 1500 cm^{-1}; NMR ($CDCl_3$, 60 MHz) 2.28 (m, 2 H, C-3 protons), 2.82 (m, 2 H, C-4 protons), 3.12 (m, 2 H, C-1 protons), 4.98 (s, 2 H, benzyl CH_2), 5.05 (s, 2 H, benzyloxy CH_2), 6.55–7.50 (m, 14 H, reducing to 13 H on addition of D_2O, NH and aromatic protons), 9.00 (br s, 1 H, replaceable with D_2O, COOH). Anal. ($C_{26}H_{25}NO_5$) C, H, N.

Preparation of N-Protected Pentapeptides. As a general method, a solution of the appropriate racemic *N*-benzyloxycarbonyl-protected amino acid (1.9 mmol), glycylglycyl-L-phenylalanyl-L-leucine methyl ester (4;[12] 1.9 mmol), and triethylamine (2.2 mmol) in CH_2Cl_2 (50 mL) was cooled in an ice bath, and 1-hydroxybenzotriazole (3.9 mol) in CH_2Cl_2 (10 mL) was added dropwise. The mixture was stirred for 5 min before adding *N*,*N*′-dicyclohexylcarbodiimide (2.1 mol) in CH_2Cl_2 (20 mL) in one portion. The ice bath was removed, the reaction was stirred at room temperature for 56 h, the precipitated dicyclohexylurea was removed by filtration, and the filtrate was evaporated to a volume of 10 mL and then refiltered. The resulting filtrate was evaporated to dryness, dissolved in ethyl acetate (50 mL), and washed with 1 N HCl (50 mL), saturated $NaHCO_3$ solution (50 mL), and finally water (50 mL). The organic layer was then dried and filtered, and the filtrate was evaporated to dryness. The resulting gummy residue was triturated with diethyl ether to afford the appropriate N-protected pentapeptide as a buff-colored, amorphous solid, consisting of a mixture of diastereoisomers.

[[5-(Benzyloxy)-2-[(benzyloxy)carbamido]-2-indanyl]carbonyl]glycylglycyl-L-phenylalanyl-L-leucine methyl ester (2d) was prepared as above from **2c** in 74% yield: mp 75–78 °C; TLC (silica) R_f (ethyl acetate/methanol, 40:60) 0.64; IR (KCl) 3175, 3050, 3020, 2945, 2920, 2860, 1718, 1682, 1670, 1645, 1635, 1535, 1525, 1518 cm^{-1}; NMR ($CHCl_3$, 300 MHz) 0.86 [d, 6 H, leucine δ-$(CH_3)_2$], 1.57 (m, 2 H, leucine β-CH_2), 1.98 (m, 1 H, leucine γ-CH_2), 3.06 (d of d, 4 H, indan 1- and 3-protons), 3.29 (m, 2 H, phenylalanine β-CH_2), 3.56 and 3.61 (2 s, 3 H, leucine OCH_3), 3.72 (m, 2 H, one of glycine α-CH_2's), 3.85 (m, 2 H, one of glycine α-CH_2's), 4.54 (m, 1 H, leucine α-CH), 4.68 (m, 1 H, phenylalanine α-CH), 5.01 (d, 4 H, 2 benzyloxy CH_2's), 6.72–7.45 (m, 18 H, aromatic protons), 6.82 (d, 1 H, replaceable with D_2O, NH), 7.20 (m, 2 H, replaceable with D_2O, 2 NH), 7.72 (m, 1 H, replaceable with D_2O, NH), 8.30 (m, 1 H, replaceable with D_2O, NH). Anal. ($C_{46}H_{51}N_5O_9$) C, N; H: calcd, 6.4; found, 6.9.

[[6-(Benzyloxy)-2-[(benzyloxy)carbamido]-2-tetralinyl]carbonyl]glycylglycyl-L-phenylalanyl-L-leucine methyl ester (3d) was prepared as above from **3c** in 89% yield: mp 78–81 °C; TLC (silica) R_f (chloroform/methanol/acetic acid, 120:90:5) 0.68; IR (KCl) 3265, 3060, 3020, 2950, 2920, 2855, 1758, 1688, 1675, 1655, 1545, 1525 cm^{-1}; NMR ($CDCl_3$, 300 MHz) δ 0.88 [d, 6 H, leucine δ-$(CH_3)_2$], 1.29 (m, 2 H, leucine β-CH_2), 1.56 (m, 1 H, leucine γ-CH_2), 1.94 (m, 2 H, tetralin 3-protons), 3.12 (m, 4 H, tetralin 1- and 4-protons), 3.34 (m, 2 H, phenylalanine β-CH_2), (s, 3 H, leucine OCH_3), 3.87 (m, 2 H, one of glycine α-CH_2's), 3.93 (m, 2 H, one of glycine α-CH_2's), 4.51 (m, 1 H, leucine α-CH), 4.72 (m, 1 H, phenylalanine α-CH), 5.02 (m, 4 H, benzyloxy CH_2's), 6.32 (m, 1 H, replaceable with D_2O, NH), 6.74–7.60 (m, 18 H, aromatic protons), 7.12 (m, 2 H, replaceable with D_2O, 2 NH), 7.80 (m, 1 H, replaceable with D_2O, NH), 8.31 (m, 1 H, replaceable with D_2O, NH). Anal. ($C_{46}H_{53}N_5O_9$) C, H, N.

Synthesis of Unprotected Pentapeptides. As a general method, the appropriate N-protected pentapeptide (16 nmol) was dissolved in absolute ethanol (100 mL), 5% palladium on charcoal catalyst (0.5 g) was added, and the mixture was hydrogenated, with stirring, at room temperature and atmospheric pressure for 20 h. The catalyst was then removed by filtration, the filtrate was evaporated to dryness, and the residue was dissolved in ethyl acetate (50 mL). The organic solution was extracted with 1 N hydrochloric acid (2 × 50 mL), followed by water (1 × 30 mL). The combined aqueous extracts were basified to pH 9 with solid sodium bicarbonate and back extracted with ethyl acetate (2 × 50 mL). The combined ethyl acetate extracts were dried and filtered, the filtrate was evaporated to dryness, and the residue was purified on a carboxymethylcellulose column, eluting with ammonium acetate buffer, as previously described, to afford the

appropriate N-deprotected pentapeptide in buffer solution. The water and buffer salts were removed by freeze–drying, redissolving the residue in water (200 mL), and redrying on an Edwards EF03 freeze–drying apparatus as 10^{-1} to 10^{-2} torr.

[(2-Amino-5-hydroxy-2-indanyl)carbonyl]glycylglycyl-L-phenylalanyl-L-leucine methyl ester (2e) was prepared as above from **2d** in 13% yield: mp 173–176 °C; TLC (silica) R_f (chloroform/methanol/acetic acid, 120:90:5) 0.68; IR (KCl) 3395, 3358, 3315, 3280, 3080, 3035, 2960, 2925, 2860, 1750, 1690, 1652, 1635, 1585, 1558, 1540, 1525, 1508 cm^{-1}; NMR ($CDCl_3$, 220 MHz) 0.82 [m, 6 H, leucine δ-$(CH_3)_2$], 1.50 (m, 2 H, leucine β-CH_2), 1.95. (m, 1 H, leucine γ-CH), 2.62 (m, 2 H, phenylalanine β-CH_2), 2.96 (m, 4 H, indan 1- and 3-protons), 3.62 (s, 3 H, leucine OCH_3), 3.65 (br s, replaceable with D_2O, NH_2 and OH), 3.69 (m, 2 H, one of glycine α-CH_2's), 3.75 (m, 2 H, one of glycine α-CH_2's), 4.24 (m, 1 H, leucine α-CH), 4.60 (m, 1 H, phenylalanine α-CH), 6.50–7.16 (m, 8 H, aromatic protons), 8.03 (m, 2 H, replaceable with D_2O, 2 NH), 8.34 (m, 1 H, replaceable with D_2O, NH) 8.42 (m, 1 H, replaceable with D_2O, NH). Anal. ($C_{30}H_{39}N_5O_7$) C, H, N; amino acid (after acidic hydrolysis): **2a**, 0.94; Gly, 2.03; Phe, 1.01; Leu, 1.00.

[(2-Amino-6-hydroxy-2-tetralinyl)carbonyl]glycylglycyl-L-phenylalanyl-L-leucine methyl ester (3e) was prepared as above from **3d** in 34% yield: mp 100–104 °C; TLC (silica) R_f (chloroform/methanol/acetic acid, 120:90:5) 0.70; IR (KCl)) 3270, 3055, 3020, 2950, 2930, 2860, 1740, 1690, 1675, 1645, 1565, 1546, 1500 cm^{-1}; NMR ($CDCl_3$, 300 MHz) 0.80 and 0.85 [2 d, 6 H, leucine δ-$(CH_3)_2$], 1.33 (m, 2 H, leucine β-CH_2), 1.51 (m, 1 H, leucine γ-CH), 1.56 (m, 2 H, tetralin 3-protons), 3.06 (m, 2 H, phenylalanine β-CH_2), 3.07 (m, 4 H, tetralin 1- and 4-protons), 3.65 (s, 3 H, leucine OCH_3), 3.82 (m, 2H, one of glycine α-CH_2's), 3.93 (m, 2 H, one of glycine α-CH_2's), 4.30 (br s, 3 H, replaceable with D_2O, NH_2 and OH), 4.47 (m, 1 H, leucine α-CH), 4.70 (m, 1 H, phenylalanine α-CH), 6.56–7.40 (m, 8 H, aromatic protons), 7.00 (m, 2 H, replaceable with D_2O, 2 NH), 7.42 (m, 1 H, replaceable with D_2O, NH), 8.43 (m, 1 H, replaceable with D_2O, NH). Anal. ($C_{31}H_{41}N_5O_7$) C, H, N; amino acid (after acidic hydrolysis): **3a** no color reaction with ninhydrin; Gly, 2.08; Phe, 1.00; Leu, 1.00.

Pharmacology. Compounds were evaluated for analgesic properties in albino mice (Tuck, TFW strain) by the following procedures: in vitro testing was carried out by measuring the inhibition of electrically stimulated contractions of the guinea pig ileum myenteric plexus muscle using the method of Kosterlitz and Watt[13] and by measuring the inhibition of mouse vas deferens tissue after stimulation wth twin rectilinear pulses 10-ms apart.[14] In vivo evaluation was carried out by using the mouse hot-plate test.[15]

Acknowledgment. We gratefully acknowledge the support of the Wellcome Research Laboratories in carrying out and releasing results of the biological testing and the Pharmaceutical Society of Great Britain for providing a research studentship for T.D.

Registry No. **2a**, 33709-81-8; **2b**·HCl, 84802-81-3; **2c**, 84802-82-4; **2d**, 84802-83-5; **2e**, 73309-72-5; **3a**, 84809-70-1; **3b**·HCl, 84802-84-6; **3c**, 84802-85-7; **3d**, 84802-86-8; **3e**, 73301-07-2; **4**, 68709-94-4; benzyl bromide, 100-39-0; carbobenzyloxy chloride, 501-53-1.

(13) H. W. Kosterlitz and A. J. Watt, *Br. J. Pharmacol. Chemother.*, **33**, 266 (1968).
(14) J. Hughes, H. W. Kosterlitz, and F. M. Leslie, *Br. J. Pharmacol.*, **53**, 371 (1975).
(15) N. B. Eddy and D. Leimbach, *J. Pharmacol. Exp. Ther.*, **107**, 385 (1953).

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Synthesis and Stereochemical Analysis of 2-Amino-1,2,3,4-tetrahydro-1,4-methanonaphthalene-2-carboxylic Acid, A Conformationally Rigid Phenylalanine Derivative

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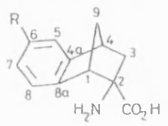
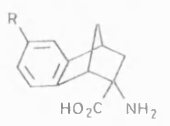
A rigid phenylalanine analogue, 2-amino-1,2,3,4-tetrahydro-1,4-methanonaphthalene-2-carboxylic acid (**3**) of unknown stereochemistry was obtained as the sole amino acid product from a Strecker reaction with 1,2,3,4-tetrahydro-1,4-methanonaphthalen-2-one (**4**). Compound (**4**) was initially treated with benzylamine and potassium cyanide to give 2-benzylamino-2-cyano-1,2,3,4-tetrahydro-1,4-methanonaphthalene (**5**), which was then converted into 2-benzylamino-2-carboxamido-1,2,3,4-tetrahydro-1,4-methanonaphthalene (**6**) by treatment with 70% sulphuric acid. *N*-Debenzylation of (**6**) by hydrogenolysis with 5% palladium-on-charcoal catalyst afforded 2-amino-2-carboxamido-1,2,3,4-tetrahydro-1,4-methanonaphthalene (**7**) which gave the acid (**3**) on heating in 10% sulphuric acid. A stereochemical analysis of (**3**) by ^{1}H n.m.r., ^{13}C n.m.r., and auto-correlated two-dimensional n.m.r. spectroscopy, determined the structure to be 2-*endo*-amino-1,2,3,4-tetrahydro-1,4-methanonaphthalene-2-*exo*-carboxylic acid (**1a**). Treatment of 1,2,3,4-tetrahydro-1,4-methanonaphthalene-2-spiro-5′-hydantoin (**9**) [obtained from a Bucherer reaction with (**4**)] with aqueous barium hydroxide also afforded (**1a**) as the only amino acid product.

Structure–activity studies recently carried out in this laboratory have focused on the biological properties of rigid phenylethylamine systems in order to examine the importance of molecular conformation on the biological activity. We have shown that 2-amino substituted 1,2,3,4-tetrahydro-1,4-methanonaphthalenes represent rigid, pharmacologically interesting analogues of more conformationally flexible phenylethylamine compounds such as amphetamine,[1] dopamine,[2] and noradrenaline,[3] which may provide useful information about the nature of agonist–receptor interactions in these phenylethylamine systems. Recently, our interest has been directed towards the synthesis of enkephalin derivatives containing a conformationally restrained *N*-terminal tyrosine residue, and we have shown[4] that replacing the L-tyrosyl moiety in Leu-enkephalin methyl ester with a (2-amino-6-hydroxy-1,2,3,4-tetrahydronaphthalenyl)-2-carbonyl grouping, leads to a 7 to 8 times higher agonist activity at the analgesic μ-receptor subtype in guinea pig ileum, when compared to Leu-enkephalin, and an almost 30-fold decrease in potency, *versus* Leu-enkephalin, on mouse vas deferens preparation, a tissue in which the δ-receptor predominates. The completely rigid aromatic amino acids (**1a**) and (**2a**), and (**1b**) and (**2b**), thus represent conformationally defined analogues of phenylalanine and tyrosine, respectively, which should be of potential value in determining the effect of completely restricting the conformational flexibility of the tyrosyl moiety in the enkephalins on receptor recognition.

This present report describes the synthesis and stereochemical analysis of 2-amino-1,2,3,4-tetrahydro-1,4-methanonaphthalene-2-carboxylic acid (**3**); this was obtained from a Strecker synthesis, starting from the previously reported ketone (**4**), and afforded only one of the two expected stereoisomers of (**3**) from this reaction sequence.



(**1**)
a; R = H
b; R = OH

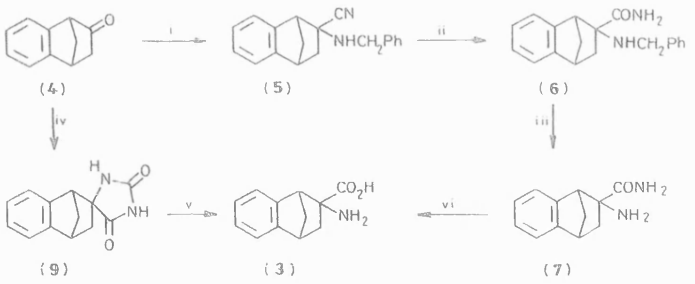


(**2**)
a; R = H
b; R = OH

Results and Discussion

Treatment of 1,2,3,4-tetrahydro-1,4-methanonaphthalen-2-one (**4**) with benzylamine and KCN in aqueous ethanolic HCl gave a pure stereoisomer (**5**) in 86% yield. No other isomeric product could be detected in the reaction mixture. This product could be hydrolysed to the carboxamido derivative (**6**) in 70% H_2SO_4 solution. Hydrogenolytic *N*-debenzylation of compound (**6**) was carried out in ethanolic glacial acetic acid, using palladium–charcoal (5%) as catalyst, and afforded the product (**7**) in 45% yield. Hydrolysis of compound (**7**) in 10% H_2SO_4 solution, heated at reflux for 5 h, afforded 2-amino-1,2,3,4-tetrahydro-1,4-methanonaphthalene-2-carboxylic acid (**3**) in 79% yield (Scheme).

In a rigid molecule such as (**3**) the Karplus relationship[5] can be applied to interpret the n.m.r. spectra and elucidate the stereochemistry at C-2. It is clear from Dreiding models of the two possible isomers of the acid (**3**) that the largest $^3J_{CH}$ vicinal coupling of the carbonyl carbon should be to the 3-H which is *cis* to it. If the carboxy group has the *exo* geometry as in compound (**1a**) this coupling would be to the *exo* 3-H, while if it is *endo* as in (**2a**), then coupling would be to the *endo* 3-H; 1-H is not at a favourable angle for coupling to the carbonyl carbon in either configuration of the carboxy group. Selective decoupling of the doublet of doublets centred at 3.04 p.p.m. in the ^{1}H n.m.r. spectrum of compound (**3**) causes the carbonyl doublet in the proton coupled ^{13}C n.m.r. spectrum to collapse to a singlet (see Figure 1). Hence, the problem of assigning the configuration of the carboxy and amino groups in the acid (**3**) is narrowed down to being able to unequivocally assign the *exo* and *endo* 3-H.



Scheme. *Reagents:* $PhCH_2NH_2$, KCN; ii, H_2SO_4; iii, Pd-C 5%, EtOH; iv, NH_4CO_3, KCN; v, $Ba(OH)_2$; vi, 10% H_2SO_4

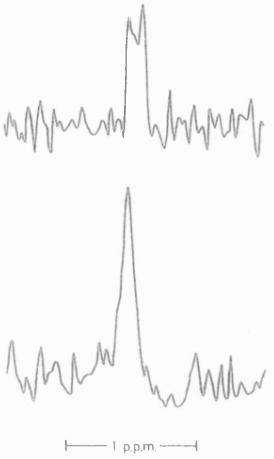


Figure 1. ^{13}C N.m.r. spectrum of compound (**3**) in $(CD_3)_2SO$–DCl with the scale expanded in the region of 173 p.p.m., (*a*) showing the ^3J(CH) vicinal coupling of the carbonyl carbon, and (*b*) showing collapse of the ^3J(CH) coupling after selective decoupling of the doublet of doublets centred at 3.04 p.p.m. in the ^1H n.m.r. spectrum of (**3**)

The aliphatic region of the ^1H n.m.r. spectrum of (**3**) in deuteriotrifluoroacetic acid ([$^2H_2$]-TFA) shows resonances in six regions, each of these signals integrating for one proton. These regions consist of a doublet of doublets (*J* 2.78, 13.9 Hz) with a central chemical shift of 3.04 p.p.m., a broad doublet centred at 2.67 p.p.m. (*J* 10.31 Hz), a doublet with unresolved fine structure centred at 2.18 p.p.m. (*J* 10.31 Hz), and a doublet of doublets centred at 1.79 p.p.m. (*J* 3.96 Hz, 13.9 Hz), and two broad singlets at 3.95 and 3.70 p.p.m. The methylenes at C-3 and C-9 should each have large geminal coupling and give rise to AB patterns. Only vicinal couplings are possible at the bridgeheads, and of these the coupling from 4-H to the *exo* 3-H should be the largest because of the small dihedral angle between these hydrogens.

An auto-correlated two-dimensional (2-D) n.m.r. experiment[6] was run and a contour plot of the results is shown in Figure 2. Two AB patterns are readily identifiable from the off-diagonal peaks. One consists of the resonances at 3.04 and 1.79 p.p.m.; the other shows the resonances at 2.67 and 2.18 p.p.m. There is a coupling of 3.79 Hz from the bridgehead signal at 3.70 p.p.m. to the doublet at 3.04 p.p.m. This is the largest coupling of the bridgehead hydrogens and the doublet at 3.04 p.p.m. is therefore assigned to the *exo* 3-H. The resonance at 1.79 p.p.m. is assigned to the *endo* 3-H leaving the remaining pair of resonances attributable to the 9-H's. These can be distinguished by further analysis of the 2-D experiment which shows a coupling between the *endo* 3-H at 1.79 p.p.m. and the resonance at 2.18 p.p.m. of 3.96 Hz. This would be the expected long range W-coupling from the *endo* 3-H to the *syn* 9-H, leaving the remaining resonance at 2.67 p.p.m. to be assigned to the *anti* 9-H. Because the *syn* 9-H is juxtaposed over the aromatic ring, whereas the *anti* 9-H is not, the ring anisotropy would tend to push the chemical shift of the *syn* 9-H more upfield than the *anti* 9-H.

Since the carbonyl carbon resonance collapses to a singlet on irradiation of the 3.04 p.p.m. resonance in the ^1H n.m.r. spectrum, and is unaffected upon irradiation of the 1.79 p.p.m. resonance, the carboxy group must be in the *exo*-configuration, and the stereochemistry of compound (**3**) is as indicated in structure (**1a**). The large downfield shift of the *exo* 3-H is probably due to a preference for the conformation in which it lies in the plane of the carbonyl group. The shift assignments and couplings described above and additional confirmation from 2-D *J* spectra[6] are summarized in the Table.

The assignment of *exo*-stereochemistry to the 2-carboxy group in (**3**) indicates that in the initial reaction of (**4**) with benzylamine, attack of cyanide ion on the resulting imino intermediate (**8**) occurs at the *exo*-face of the molecule to give the kinetically more favourable 2-*endo*-benzylamino-2-*exo*-cyano stereoisomer of (**5**). From similar reactions with other cyclic ketones,[7–10] including norbornanone[9] and the related ketone, 1,4-dihydro-1,4-ethanonaphthalen-2(3*H*)-one[11] (**10**), mixtures of both *exo*- and *endo*-amino acids are obtained. The present results obtained with compound (**4**) are interesting in that, to our knowledge, they represent the first example of stereochemical specificity in a Strecker reaction involving a cyclic ketone.

We have also observed that the acid (**1a**) is the only isomeric product obtained from the Bucherer reaction of compound (**4**) with ammonium carbonate and KCN followed by barium hydroxide hydrolysis of the intermediate spirohydantoin (**9**). Again, these results are somewhat surprising in view of a report on the formation of nearly equal mixtures of isomeric hydantoins from the reaction of (**10**)[11] with ammonium carbonate and KCN under similar conditions. So far, we have been unable to detect the presence of the isomeric amino acid (**2a**) in the reaction products from either of the above synthetic routes.

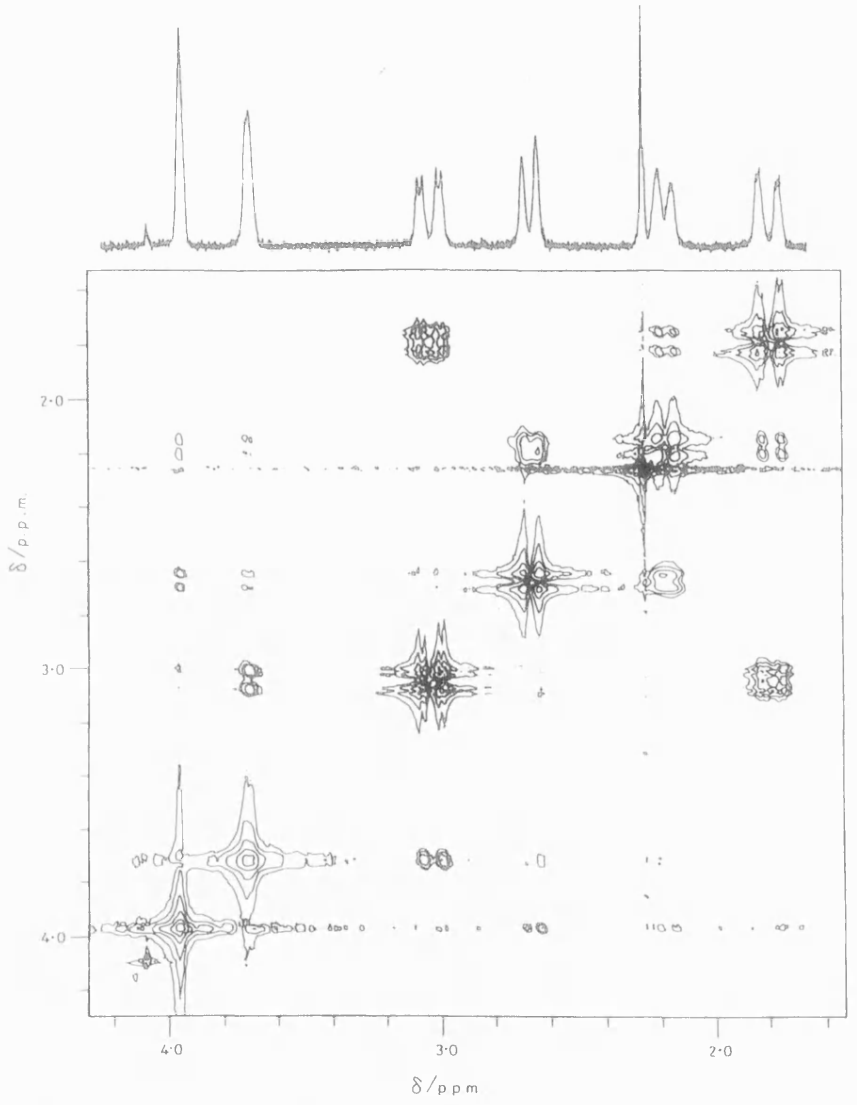
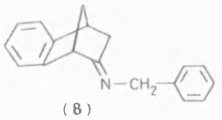
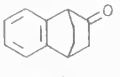


Figure 2. 200 MHz Auto-correlated two-dimensional ^{1}H n.m.r. spectrum of compound (3) in TFA



(8)



(10)

Experimental

M.p.s were measured on a Reichert hot-stage microscope. Recrystallization solvents are shown in parentheses. Yields of solids refer to products obtained prior to recrystallization. I.r. spectra were recorded on a Perkin-Elmer model 237 grating spectrophotometer.

^{1}H N.m.r. spectra were recorded on Perkin-Elmer R24 (60 MHz) or Varian XL-200 (200 MHz) spectrometers; ^{13}C n.m.r. spectra (50.3 MHz) and two dimensional n.m.r. spectra were recorded on a Varian XL-200 spectrometer. As expected, the n.m.r. parameters are solvent and pH dependent. Microanalyses were conducted by Mr. M. Hart, Department of Chemistry, Manchester.

Table. N.m.r. parameters for the acid (3)

δ_H ([$^2H_1$]-TFA)		$J_{^1H}$/Hz ([$^2H_1$]-TFA)		δ_C/p.p.m. [$(CD_3)_2SO$-DCl]	
1-H	3.15	$^2J_{2\text{-}exo,3\text{-}endo}$	13.9(3)	C-1	52.4
3-*exo*-H	3.04	$^2J_{9\text{-}anti,9\text{-}syn}$	10.30	C-2	63.8
3-*endo*-H	1.79	$^3J_{3\text{-}exo,4}$	3.78	C-3	49.4
4-H	3.15	$^4J_{3\text{-}endo,9\text{-}syn}$	3.96	C-4	43.5
9-*anti*-H	2.67	$^3J_{3\text{-}endo,4}$	(0.48)	C-4a	141.0
9-*syn*-H	2.18	$^3J_{1,9\text{-}syn}$	1.26	C-5[a]	127.0
		$^3J_{1,9\text{-}anti}$	1.76	C-6[b]	122.3
		$^3J_{4,9\text{-}anti}$	1.76	C-7[b]	124.8
				C-8[a]	128.5
				C-8a	148.5
				C-9	67.0
				C=O	172.9

[a,b] Signals may be interchanged.

2-*Benzylamino*-2-*cyano*-1,2,3,4-*tetrahydro*-1,4-*methanonaphthalene* (5).—1,2,3,4-Tetrahydro-1,4-methanonaphthalen-2-one (4)[12] (1 g, 6.0 mmol) in ethanol (20 ml) was added to a solution of benzylamine (0.75 g, 7.0 mmol) in water (20 ml), made just acid with dilute HCl. The solution was cooled to 0 °C in an ice–sodium chloride bath, and KCN (0.49 g, 8.0 mmol) in water (20 ml) was added dropwise, during 5 min, with vigorous stirring. The cooling bath was removed and the solution stirred at room temperature for 24 h, during which time the oily precipitate, formed after addition of the KCN, had crystallized. The crystals were collected by filtration affording 2-*benzylamino*-2-*cyano*-1,2,3,4-*tetrahydro*-1,4-*methanonaphthalene* (5) (1.49 g, 86%), m.p. 93—100 °C, $\nu_{max.}$ (KCl) 2 210 cm^{-1} (C≡N); δ($CDCl_3$) 1.12 (1 H, s, exchangeable with D_2O, NH), 1.21 (1 H, d of d, *J* 3 and 12 Hz, *exo* 3-H), 2.09 (2 H, m, 9-H), 2.63 (1 H, d of d, *J* 4 and 12 Hz, *endo* 3-H), 3.36 (1 H, m, 1-H), 3.77 (1 H, m, 4-H), 3.83 (2 H, s, benzyl CH_2), and 6.96—7.50 (9 H, m, aromatic H) (Found: C, 83.2; H, 6.5; N, 10.2. $C_{19}H_{18}N_2$ requires C, 83.2; H, 6.6; N, 10.2%).

2-*Benzylamino*-2-*carboxamido*-1,2,3,4-*tetrahydro*-1,4-*methanonaphthalene* (6).—Compound (5) (1.3 g) was stirred with 70% H_2SO_4 (20 ml) at room temperature for 1 h and the mixture poured onto crushed ice (100 g). When all the ice had melted the resulting mixture was filtered and the filtrate adjusted to pH 3 with 5M-NaOH solution. A white precipitate was formed, which was collected by filtration at the pump and crystallized from diethyl ether to afford 2-*benzylamino*-2-*carboxamido*-1,2,3,4-*tetrahydro*-1,4-*methanonaphthalene* (6) (950 mg, 69%), m.p. 145—148 °C, $\nu_{max.}$ (KCl) 1 655 and 5 858 cm^{-1}, δ($CDCl_3$) 1.20 (1 H, d of d, *endo* 3-H), 2.22 (2 H, m, 9-H), 2.70 (1 H, d of d, *exo* 3-H), 3.31 (1 H, m, 1-H), 3.57 (2 H, d of d, benzyl CH_2), 3.69 (1 H, m, 4-H), 6.08 (3 H, br s, NH), 6.84—7.55 (4 H, m, ArH), and 7.14 (5 H, s, aromatic H of benzyl group) (Found: C, 77.6; H, 6.9; N, 9.2. $C_{19}H_{20}N_2O \cdot 1/8H_2O$ requires C, 77.5; H, 6.9; N, 9.5%). (This trace of water was not removable on prolonged drying.)

2-*Amino*-2-*carboxamido*-1,2,3,4-*tetrahydro*-1,4-*methanonaphthalene* (7).—Compound (6) (8.9 g) was dissolved in absolute ethanol (100 ml), and palladium–charcoal catalyst (5%, 2 g) was added. Glacial acetic acid (20 ml) was added to the reaction mixture, and the mixture was hydrogenated with stirring at room temperature and atmospheric pressure overnight, until no further uptake of hydrogen was observed. The catalyst was removed by filtration and washed with hot 96% ethanol. The combined filtrate and washings were evaporated to dryness and the residue partitioned between 5M-HCl (200 ml) and ethyl acetate (100 ml). The acidic aqueous layer was separated, basified with 4M-NaOH solution to pH 9 and extracted with ethyl acetate (2 × 100 ml). There remained some insoluble material in the aqueous layer which was collected by filtration. The combined ethyl acetate extracts were dried, filtered, evaporated to dryness, and the residue crystallized from diethyl ether. Both the insoluble material and the crystallized material from the extract were identified as 2-*amino*-2-*carboxamido*-1,2,3,4-*tetrahydro*-1,4-*methanonaphthalene* (7) (2.79 g, 45%), m.p. 174—177 °C, $\nu_{max.}$ (KCl) 3 350 (NH_2), 1 665, 1 653, 1 575, 1 560, and 1 540 cm^{-1}; δ($CDCl_3$) 0.93 (1 H, d of d, *endo* 3-H), 2.20 (2 H, m, 9-H), 2.96 (1 H, d of d, *exo* 3-H), 3.37 (1 H, m, 1-H), 4.63 (1 H, m, 4-H), 5.75 (4 H, br s, NH), and 6.55—7.90 (4 H, m, aromatic H) (Found: C, 67.8; H, 6.8; N, 12.9. $C_{12}H_{14}N_2O$ requires C, 68.2; H, 7.1; N, 13.2%).

1,2,3,4-*Tetrahydro*-1,4-*methanonaphthalene*-2-*spiro*-5′-*hydantoin* (9).—A solution of ammonium carbonate (28.6 g, 0.18 mol) in 50% aqueous ethanol (200 ml) was added to a solution of (4) (11.1 g, 0.07 mol) in 50% aqueous ethanol (40 ml), in a two-necked flask fitted with a reflux condenser and a dropping funnel. The flask was warmed to 50 °C in an oil-bath and a solution of KCN (4.6 g, 0.072 mol) in water (40 ml) was added in small portions during 1 h. The reaction mixture was stirred magnetically and the temperature held at 58—60 °C for 5 h. The ethanol was then removed by evaporation and the aqueous mixture was allowed to cool and extracted with ethyl acetate (3 × 200 ml). The combined organic extracts were dried, filtered, and evaporated to dryness and the residue was triturated with diethyl ether to afford white crystals of 1,2,3,4-*tetrahydro*-1,4-*methanonaphthalene*-2-*spiro*-5′-*hydantoin* (9), m.p. 225—229 °C (methanol–diethyl ether); $\nu_{max.}$ (KCl) 1 785, 1 775, and 1 725 cm^{-1}; δ[$(CD_3)_2SO$] 1.28 (1 H, d of d, *J* 5 and 14 Hz, *endo* 3-H), 1.63 (1 H, m, *syn* 9-H), 2.22—2.80 (2 H, m, *anti* 9-H and *exo* 3-H), 3.38—3.57 (2 H, m, 1-H and 4-H), 6.77 (1 H, br s, exchangeable with D_2O, 1′-NH), 7.18 (4 H, m, aromatic H), and 10.60 (1 H, br s, exchangeable with D_2O, 3′-NH) (Found: C, 68.3; H, 5.4; N, 11.8. $C_{13}H_{12}N_2O_2$ requires C, 68.4; H, 5.2; N, 12.3%). The aqueous layer was carefully acidified to pH 2 with concentrated H_2SO_4 to afford a further crop of the above product (total yield 12.07 g, 76%).

2-endo-*Amino*-1,2,3,4-*tetrahydro*-1,4-*methanonaphthalene*-2-exo-*carboxylic Acid* (1a).—*Method a.* A solution of (7) (2.5 g) in 10% H_2SO_4 (100 ml) was heated at reflux, for 5 h. The reaction mixture was then filtered while still hot and the filtrate was cooled in an ice-bath, basified to pH 6 with 4M-NaOH solution, evaporated under reduced pressure to a low volume (*ca.* 20 ml), and allowed to cool to afford white crystals of 2-endo-*amino*-1,2,3,4-*tetrahydro*-1,4-*methanonaphthalene*-2-exo-*carboxylic acid* (1a) (1.98 g, 79%) m.p. 227.5—229.5 °C, $\nu_{max.}$ (KCl) 1 660, 1 645, 1 625, and 1 540 cm^{-1}; δ(D_2O; 60 MHz) 1.48 (1 H, d of d, *J* 4 and 14 Hz, *endo* 3-H), 2.25 (2 H, m, 9-H), 2.95 (1 H, d of d, *J* 4 and 14 Hz, *exo* 3-H), 3.58 (1 H, m, 1-H), 3.76 (1 H, m, 4-H), and 7.37 (4 H, m, aromatic H). The *hydrochloride salt* was prepared from a portion of the product and had m.p. 228—231 °C (decomp.) (Found: C, 67.8; H, 6.8; N, 12.9. $C_{12}H_{13}NO_2 \cdot HCl \cdot 1/2H_2O$ requires C, 68.2; H, 7.1; N, 13.2%).

Method b. Compound (9) (6.63 g, 0.029 mol), barium hydroxide [$Ba(OH)_2 \cdot 8H_2O$] (17.6 g, 0.056 mol), and water (100 ml) were placed in a two-necked flask fitted with a nitrogen inlet and a reflux condenser, and the mixture was heated under reflux under nitrogen for 70 h. The mixture was then filtered while hot, and the collected solid washed with an equal volume of water. The combined filtrate and washings were saturated with carbon dioxide, heated to the boiling point, and refiltered. The filtrate on cooling afforded white crystals of (9) (140 mg, 2% returned). The mother-liquors were evaporated to *ca.* 50 ml to afford white crystals (4.69 g, 80%), which had identical spectral properties

(i.r., ^{1}H n.m.r., and ^{13}C n.m.r. spectra) with (1a) prepared *via* method a above.

References

1 P. Burn, P. A. Crooks, B. L. Ratcliffe, and J. M. H. Rees, *J. Pharm. Pharmacol.*, 1980, **32**, 87.
2 P. Burn, P. A. Crooks, F. Heatley, B. Costall, R. J. Naylor, and V. Nohria, *J. Med. Chem.*, 1982, **25**, 363.
3 P. Hicks, C. Waldron, P. Burn, and P. A. Crooks, *J. Pharm. Pharmacol.*, 1980, **35**, 94.
4 T. Deeks, P. A. Crooks, and R. D. Waigh, *J. Med. Chem.*, 1983, **26**, 762.
5 M. Karplus, *J. Chem. Phys.*, 1959, **30**, 1959.
6 A. Bax, 'Two Dimensional Nuclear Magnetic Resonance in Liquids,' Delft University Press, D. Reidel Publishing Company, London, 1982.
7 K. Tori, K. Aono, Y. Hata, R. Muneyuki, T. Tsuji, and H. Tanida, *Tetrahedron Lett.*, 1966, 9.
8 Y. Maki and T. Magsugi, *Chem. Pharm. Bull.*, 1973, **21**, 685.
9 N. S. Trager and H. N. Christensen, *J. Am. Chem. Soc.*, 1972, **94**, 968.
10 G. G. Trigo, C. Avendano, S. Emila, J. T. Edward, and S. Wong, *Can. J. Chem.*, 1979, **57**, 754.
11 G. L. Grunewald, S. H. Kuttal, M. A. Pleiss, J. B. Mangold, and P. Soine, *J. Med. Chem.*, 1980, **23**, 754.
12 D. J. Sandman, K. Mislow, W. P. Giddings, J. Dirlam, and G. C. Hanson, *J. Am. Chem. Soc.*, 1968, **90**, 4877.

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Leucine Enkephalin Analogues Containing a Conformationally Restrained *N*-Terminal Amino Acid Residue

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Abstract □ Three analogues of leucine enkephalin, in which the terminal tyrosine-1 residue has been replaced by conformationally restrained aromatic amino acids, have been synthesized by classical solution methods. Their opiate agonist potencies on electrically stimulated guinea pig ileum and mouse vas deferens preparations were determined and compared with morphine, Met enkephalin, and Leu enkephalin. None of these analogues had analgesic properties when evaluated on the above tissue preparations or when evaluated by the hot-plate test in mice after subcutaneous and intracerebroventricular administration.

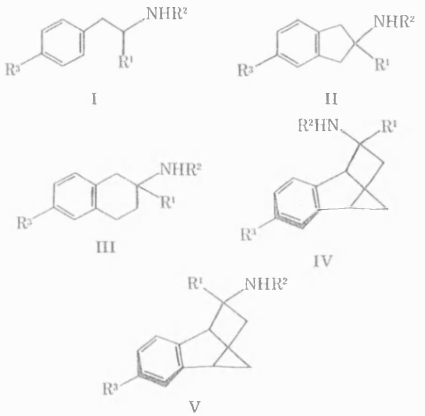
Keyphrases □ Leucine enkephalin—conformationally restrained analogues, synthesis, analgesic activity □ Analgesics—evaluation of conformationally restrained leucine enkephalin derivatives, synthesis

A milestone in neurochemical research during the last decade has been the elucidation of the role of enkephalins as natural endogenous ligands for the opiate receptor (1, 2). Since this discovery, the synthesis of a multitiude of structural analogues of leucine and methionine enkephalins (Ig and Ih, respectively) have been reported (recent review, 3), some of which are more potent analgesics and/or possess greater *in vivo* stability than the natural opiate ligands. These synthetic analogues have resulted from modifications of the parent molecules, such as shortening or lengthening of the pentapeptide chain (4, 5), substitution of individual amino acids by other amino acids (5–11), and chemical modification of individual amino acids (12–14).

Preferred conformations of enkephalin molecules at analgesic receptors have been suggested, based on data from conformational studies involving both spectroscopic measurements and structure–activity considerations (15–33). These studies have attempted to relate the structure of the terminal tyrosine residue in the enkephalin molecule to the tyramine moiety present in the morphine molecule. Since morphine is a conformationally rigid molecule, this implies that the conformationally "loose" tyrosine residue in the enkephalin may interact at opiate receptors in a specific conformation which is related, stereochemically, to the rigid tyramine moiety present in the morphine molecule.

As part of a study designed to evaluate the importance of the tyrosine conformation in the enkephalins on analgesic activity and receptor recognition, we have initiated a preliminary investigation into the synthesis of Leu enkephalin derivatives in which the terminal tyrosine unit has been replaced by a variety of conformationally restrained aromatic amino acids and in which the terminal leucine-5 residue has been esterified to aid passage into the central nervous system. The investigations described in this report are restricted to the preparation of Leu enkephalin analogues containing a nonhydroxylated aromatic amino acid in place of the tyrosine-1 residue in order to initially determine the feasibility of preparing pentapeptides such as IIe–Ve, with a bulky terminal amino acid unit, *via* classical solution methods, before embarking on the preparation of the synthetically more difficult aromatic hydroxy derivatives IIf–Vf, which were regarded as the ultimate target compounds.

We now report the synthesis of the Leu enkephalin analogues IIe–IVe and their evaluation as analgesic agents on isolated guinea pig ileum myenteric plexus muscle, mouse vas deferens tissue, and by the hot-plate test in mice after subcutaneous and intracerebroventricular administration.



I II III IV V

a R^1 = COOH, R^2 = R^3 = H.
b R^1,R^2 = CO—NHCO—, R^3 = H.
c R^1 = COOH, R^2 = CO—O—CH_2Ph, R^3 = H.
d R^1 = CONHGlyGlyL—PheL—LeuOCH$_3$, R^2 = CO—OCH_2Ph, R^3 = H.
e R^1 = CONHGlyGlyL—PheL—LeuOCH$_3$, R^2 = R^3 = H.
f R^1 = CONHGlyGlyL—PheL—LeuOCH$_3$, R^2 = H, R^3 = OH.
g R^1 = CONHGlyGlyL—PheL—LeuOH, R^2 = H, R^3 = OH.
h R^1 = CONHGlyGlyL—PheL—MetOH, R^2 = H, R^3 = OH.

H_2NGlyGlyL—PheL—LeuOCH$_3$

VI

EXPERIMENTAL

Melting points were determined on a hot-stage microscope[1] and are uncorrected. Microanalyses were performed by the Micro-analytical Laboratory, Department of Chemistry, University of Manchester, analytical results obtained for all compounds were within ±0.4% of the theoretical value unless otherwise stated. ^{1}H-NMR spectra were recorded on a spectrometer[2] and are quoted in ppm on the δ scale. ^{13}C-NMR spectra were recorded on a spectrometer[3]. IR spectra were recorded as KCl disks, nujol mulls, or liquid films

[1] Reichert.
[2] Model HR 220 or SC 300; Varian Instruments.
[3] Model WP 80; Bruker.

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on a spectrophotometer[4]. Mass spectra were determined on a spectrometer[5] operating at a probe temperature of 250°C. TLC separations were carried out on 0.25-mm silica gel[6]. 2-Aminoindan-2-carboxylic acid (34) and (±)-2-aminotetralin-2-carboxylic acid (35) were prepared by literature procedures. Amino acids and peptides with free amino functions were visualized by spraying with 1% ninhydrin in ethanol. *N*-Protected amino acids and peptides were visualized with 5% potassium dichromate in concentrated H_2SO_4; other compounds were visualized in iodine vapor. Hydrogenations were carried out on a hydrogenator[7] operating at atmospheric pressure and room temperature.

Ion-exchange chromatographic separations of *N*-deprotected pentapeptides were carried out on carboxymethylcellulose[8], prewashed with 0.5 M sulfuric acid followed by 0.2 M ammonium acetate buffer adjusted to pH 5.1 with 0.88 M ammonium hydroxide or glacial acetic acid and measured using a pH meter[9] fitted with a combined electrode[10]. Ion-exchange columns were run under slight pressure using a peristaltic pump set for an effluent output of 100 mL/h. Column effluents were monitored by UV spectrophotometry at 280 nm using a spectrophotometer[11] fitted with a 10-mm path length flow-through cell. Columns were eluted with 0.005–0.5 M gradient ammonium acetate buffer solutions at pH 5.1, and fractions were collected on an automatic fraction collector.

Amino acid analyses of peptides were performed on an amino acid analyzer[12] using ninhydrin as visualizer. The peptides were hydrolyzed in 6 M HCl at 110°C for 24 h in a sealed, evacuated tube.

Synthesis of *endo*-2-Aminobenzonorbornen-2-carboxylic Acid (IVa)—*Spiro[benzonorbornen-2,5′-hydantoin] (IVb)*—A solution of ammonium carbonate (28.6 g, 0.18 mol) in 50% aqueous ethanol (200 mL) was added to benzonorbornen-2-one (36) (11.1 g, 0.07 mol) in 50% aqueous ethanol (40 mL). The mixture was warmed to 50°C and a solution of potassium cyanide (4.6 g, 0.072 mol) in water (40 mL) was added in small portions over a 1-h period. The mixture was stirred at 58–60°C for 5 h. The ethanol was removed under reduced pressure, and the aqueous residue was extracted with ethyl acetate (3 × 200 mL). The combined organic extracts were dried, filtered, and evaporated to dryness, and the residue was triturated with ether to afford white crystals of IVb mp 225–229°C. The aqueous layer was acidified to pH 2 with concentrated sulfuric acid to afford an additional product for a total of 12.07 g 76%). This compound had identical spectrometric properties (IR, NMR) to an authentic sample recently prepared *via* a Strecker synthesis (48).

endo-*2-Aminobenzonorbornen-2-carboxylic Acid (IVa)*—A mixture of spiro[benzonorbornen-2,5′-hydantoin] (IVb) (6.63 g, 0.029 mol), barium hydroxide [$Ba(OH)_2{\cdot}8H_2O$, 17.6 g, 0.056 mol], and water (100 mL) was heated under reflux, in an atmosphere of nitrogen, for 70 h and then filtered while hot. The collected solid was washed with an equal volume of water, and the combined filtrate and washings were saturated with carbon dioxide, heated to boiling point, and refiltered. The filtrate (on cooling) afforded white crystals of IVb (140 mg, 2% recovery). The mother liquors were evaporated to ~50 mL to afford white crystals of IVa (4.69 g, 79%), mp 227.0–229.5°C [lit. (48) mp 227.5–229.5°C]. The hydrochloride salt was prepared from a portion of the product and had mp 228–231°C (dec.). ^{13}C-NMR (CH_3OH): 173.8 (s, CO_2H), 149.5 and 141.8 (2 × s, C-4a and C-8a), 129.5, 128.0, 125.2, and 123.1 (4 × d, ArC), 65.1 (s, C-2), 53.9 (d, C-4), 51.1 (d, C-1), 44.9 (t, C-9), and 39.8 (t, C-3); *m/z* 203 (M^+).

Preparation of *N*-Benzyloxycarbonyl Amino Acids—As a general method, the appropriate amino acid (0.011 mol) suspended in 2 M NaOH solution (100 mL) was heated on a steam bath to dissolve the amino acid and then cooled in an ice bath to 0–5°C, which caused the sodium salt of the amino acid to precipitate. Carbobenzyloxy chloride (2.11 g, 0.012 mol) was added in a dropwise manner to the cooled, stirred mixture over 40 min. The ice bath was then removed, and the mixture was stirred at room temperature for 30 h. The mixture was cooled to 0–5°C, and a further 2.11 g (0.012 mol) of carbobenzyloxy chloride added in a dropwise manner. After 60 h the mixture was carefully adjusted to pH 1 with 5 M HCl and extracted with ether (2 × 100 mL). The combined organic extracts were dried, filtered, and evaporated to dryness, and the residue was crystallized from ether–petroleum ether (bp 30–40°C) to afford the appropriate *N*-benzyloxycarbonyl amino acid.

2-Benzyloxycarbamidoindan-2-carboxylic acid (IIc)—This compound was prepared as described above from IIa in 32% yield, mp 46.5–53.0°C. IR (Nujol): 1748, 1663, 1545, and 1532 cm^{-1}; ^{1}H-NMR (DMSO-d_6): δ 3.48 (m, 4, C-1 and C-3 H), 5.04 (s, 2, benzyloxy CH_2), 7.05–7.45 (m, 9, ArH), 7.88 (s, 1, exchangeable with D_2O, NH), and 9.10 ppm (br s, 1, exchangeable with D_2O, COOH); *m/z* 311 (M^+).

Anal.—Calc. for $C_{18}H_{17}NO_4$: C, 69.5; H, 5.5; N, 4.5. Found: C, 69.9; H, 5.8; N, 4.2.

2-Benzyloxycarbamidotetralin-2-carboxylic Acid (IIIc)—Compound IIIc was prepared as described above from IIIa in 19% yield, mp 131.0–137.5°C. IR (KCl): 1772, 1728, 1688, 1680, 1580, and 1526 cm^{-1}; ^{1}H-NMR ($CDCl_3$): δ 2.24 (m, 2, C-3 H), 2.80 (m, 2, C-4 H), 3.18 (m, 2, C-1 H), 5.05 (s, 2, benzyloxy CH_2), 5.30 (br s, 1, exchangeable with D_2O, NH), 6.90–7.50 (m, 9, ArH), and 10.32 (br s, 1, exchangeable with D_2O, COOH); *m/z* 325 (M^+).

Anal.—Calc. for $C_{19}H_{19}NO_4$: C, 70.2; H, 5.8; N, 4.3. Found: C, 70.1, H, 5.9, N, 4.0.

endo-*2-Benzyloxycarbamidobenzonorbornene-2-carboxylic Acid (IVc)*—This compound was prepared as described above from IVa in 27% yield, mp 90–93°C. IR (KCl): 1742, 1686, 1641, 1600, and 1582 cm^{-1}; ^{1}H-NMR ($CDCl_3$): δ 1.28 (d of d, 1, J = 3 and 12 Hz, C-3 *endo*-H), 1.91 and 2.26 (2 × d, AB, J = 9 Hz, C-9 H), 2.95 (d of d, 1, J = 4 and 12 Hz, C-3 *exo*-H), 3.37 (m, 1, C-4 H), 3.84 (m, 1, C-1 H), 4.80 (br s, 1, exchangeable with D_2O, NH), 5.00 (d, 2, J = 4 Hz, benzyloxy CH_2), 6.95–7.50 (m, 9, ArH), and 11.05 ppm (s, 1, exchangeable with D_2O, COOH).

Anal.—Calc. for $C_{20}H_{25}NO_5$: C, 71.2; H, 5.6; N, 4.2. Found: C, 71.6; H, 5.8; N, 4.0.

Preparation of *N*-Protected Pentapeptides—As a general method, a solution of the appropriate *N*-benzyloxycarbonyl amino acid (1.9 mmol), glycylglycyl-L-phenylalanyl-L-leucine methyl ester (VI) (37) (1.9 mmol), and triethylamine (2.2 mmol) in CH_2Cl_2 (50 mL) was cooled in an ice bath, and 1-hydroxybenzotriazole (3.9 mol) in CH_2Cl_2 (10 mL) was added in a dropwise manner. The mixture was stirred for 5 min before adding *N,N′*-dicyclohexylcarbodiimide (2.1 mol) in CH_2Cl_2 (20 mL) in one portion. The ice bath was removed, the reaction was stirred at room temperature for 56 h, the precipitated dicyclohexylurea was removed by filtration, and the filtrate was evaporated to a volume of 10 mL and then refiltered. The resulting filtrate was evaporated to dryness, dissolved in ethyl acetate (50 mL) and washed with 1 M HCl (50 mL), saturated $NaHCO_3$ solution (50 mL), and finally water (50 mL). The organic layer was then dried, filtered, and evaporated to dryness. The resulting gummy residue was triturated with ether to afford the appropriate *N*-protected pentapeptide as a buff-colored, amorphous solid.

[(2 - Benzyloxycarbamido) -2- indanyl]carbonyl]glycylglycyl-L-phenylalanyl-L-leucine Methyl Ester (IId)—Compound IId was prepared as above from IIc in 61% yield, mp 134–138°C. TLC (silica, ethyl acetate–methanol, 40:60): R_f 0.60; IR (KCl): 1726, 1675, 1665, 1650, 1595, 1532, and 1520 cm^{-1}; ^{1}H-NMR ($CDCl_3$): δ 0.81 [d, 6, leucine δ-$(CH_3)_2$], 1.51 (m, 2, leucine β-CH_2), 1.78 (m, 1, leucine γ-CH), 3.07 (d of d, 4, indane 1- and 3-H), 3.09 (m, 2, phenylalanine β-CH_2), 3.55 and 3.57 (2 × s, 3, conformational forms of leucine OCH_3), 3.72 (m, 2, one of glycine α-CH_2's), 3.92 (m, 2, one of glycine α-CH_2's), 4.49 (m, 1, leucine α-CH), 4.71 (m, 1, phenylalanine α-CH), 4.99 (d, 2, $C_6H_5OCH_2$—O), 6.28 (d, 1, exchangeable with D_2O, NH), 7.03 (t, 2, exchangeable with D_2O, 2 × NH), 7.08–7.35 (m, 14, ArH), and 7.66 ppm (m, 2, exchangeable with D_2O, 2 × NH).

Anal.—Calc. for $C_{38}H_{45}N_5O_8$: C, 65.2; H, 6.2; N, 10.0. Found: C, 65.3; H, 6.2; N, 10.0.

{[(2-Benzyloxycarbamido)-2-tetralyl]carbonyl} glycylglycyl-L-phenylalanyl-L-leucine Methyl Ester (IIId)—This compound was prepared as above from IIIc in 91% yield, mp 86–90°C. TLC (silica, chloroform–methanol–acetic acid, 120:90:5): R_f 0.62; IR (KCl): 1740, 1725, 1704, 1690, 1675, 1660, 1564, and 1530 cm^{-1}; ^{1}H-NMR ($CDCl_3$): δ 0.87 [d, 6, leucine δ-$CH_3)_2$], 1.55 (m, 2, leucine β-CH_2), 2.22 (m, 1, leucine γ-CH), 2.41 (m, 2, tetralin 3-H), 2.60–3.80 (m, 4, tetralin 1- and 4-H), 3.32 (m, 2, phenylalanine β-CH_2), 3.52 and 3.54 (2 × s, 3, leucine OCH_3's), 3.80 (m, 2, one of glycine α-CH_2's), 3.91 (m, 2, one of glycine α-CH_2's), 4.54 (m, 1, leucine α-CH), 4.73 (m, 1, phenylalanine α-CH), 5.02 (d, 2, benzyloxy CH_2), 6.78–7.70 (m, 14, ArH), 6.83 (d, 1, exchangeable with D_2O, NH), 7.16 (t, 2, exchangeable with D_2O, 2 × NH), 7.78 (m, 1, exchangeable with D_2O, NH), and 7.89 ppm (m, 1, exchangeable with D_2O, NH).

Anal.—Calc. for $C_{39}H_{47}N_5O_8$: C, 65.6; H, 6.6; N, 9.8. Found: C, 65.2; H, 6.7; N, 10.1.

[(endo-2-Benzyloxycarbamido) -2- benzonorbornenyl]carbonyl]glycylglycyl-L-phenylalanyl-L-leucine Methyl Ester (IVd)—Compound IVd was prepared as above from IVc in 43% yield, mp 118–121°C. TLC (silica, chloroform–methanol–acetic acid, 120:90:5): R_f 0.72; IR (KCl): 1740, 1705, 1690, 1660, 1632, 1565, 1545, and 1520 cm^{-1}; ^{1}H-NMR ($CDCl_3$): δ 0.84 [d, 6, leucine δ-$(CH_3)_2$], 1.30 (m, 1, benzonorbornenyl C-3 *exo*-H), 1.53 (m, 2, leucine β-CH_2), 1.94 (m, 1, leucine γ-CH), 1.98 (m, 2, benzonorbornenyl C-9 H), 2.64 (m, 1, benzonorbornenyl C-3 *endo*-H), 3.09 (m, 2, phenylalanine

[4] Model 237; Perkin-Elmer.
[5] A.E.I. model M59.
[6] Polygram silica gel UV_{254}.
[7] Gallenkampf.
[8] Whatman CMC 52.
[9] Model PW 9418; Pye Unicam.
[10] Pye Unicam 401.
[11] Cecil CE 202.
[12] Model 123; Beckmann SPINCO.

β-CH_2), 3.62 (s, 3, leucine OCH_3), 3.63 (m, 2, benzonorbornenyl C-1 and C-4 H), 3.72 (m, 2, one of glycine α-CH_2's), 3.94 (m, 2, one of glycine α-CH_2's), 4.52 (m, 1, leucine α-CH), 4.69 (m, 1, phenylalanine α-CH), 5.17 (d, 2, benzyloxy CH_2), 6.38 (m, 1, exchangeable with D_2O, NH), 6.70–7.55 (m, 16, ArH and 2 exchangeable × NH), 7.60 (m, 1, exchangeable with D_2O, NH), and 7.70 ppm (m, 1, exchangeable with D_2O, NH).

Anal.—Calc. for $C_{40}H_{47}N_5O_8$: C, 66.2; H, 6.5; N, 9.2. Found: C, 65.8; H, 6.6; N, 8.9.

Synthesis of Unprotected Pentapeptides—As a general method, the appropriate *N*-protected pentapeptide (16 mmol) was dissolved in absolute ethanol (100 mL), 5% palladium-on-charcoal catalyst (0.5 g) was added, and the mixture was hydrogenated with stirring at room temperature and atmospheric pressure for 20 h. The catalyst was then removed by filtration, the filtrate evaporated to dryness, and the residue dissolved in ethyl acetate (50 mL). The organic solution was extracted with 1 M HCl (2 × 50 mL), followed by water (1 × 30 mL). The combined aqueous extracts were basified to pH 9 with solid sodium bicarbonate and back-extracted with ethyl acetate (2 × 50 mL). The combined ethyl acetate extracts were dried, filtered, and evaporated to dryness, and the residue was purified on a carboxymethylcellulose column, eluting with ammonium acetate buffer, as previously described, to afford the appropriate *N*-deprotected pentapeptide in buffer solution. The water and buffer salts were removed by freeze-drying, redissolving the residue in water (200 mL), and redrying on a freeze-drying apparatus[13] at 10^{-1}–10^{-2} torr.

[(2-Aminoindanyl)carbonyl]glycylglycyl -L- phenylalanyl -L- leucine Methyl Ester (IIe)—This compound was prepared as above from IId in 17% yield, mp 93–95°C. TLC (silica, chloroform–methanol–acetic acid, 120:90:5): R_f 0.69; IR (KCl): 1740, 1686, 1672, 1650, 1580, 1562, 1545, and 1525 cm^{-1}; ^{1}H-NMR ($CDCl_3$): δ 0.85 and 0.93 [t and d, 6, leucine δ-$(CH_3)_2$], 1.60 (m, 2, leucine β-CH_2), 2.12 (m, 1, leucine γ-CH), 2.82 (d of d, 4, indane 1- and 3-H), 3.08, (m, 2, phenylalanine β-CH_2), 3.65 and 3.70 (2 × s, 3, leucine OCH_3), 3.81 (s, 2, exchangeable with D_2O, NH_2), 3.95 (d of d, 2, one of glycine α-CH_2's), 4.06 (d of d, 2, one of glycine α-CH_2's), 4.48 (m, 1, leucine α-CH), 4.92 (t, 1, phenylalanine α-CH), 7.00–7.31 (m, 9, ArH), 7.26 (d, 1, exchangeable with D_2O, NH), 7.49 (d, 1, exchangeable with D_2O, NH), 7.63 (m, 1, exchangeable with D_2O, NH), and 8.45 ppm (m, 1, exchangeable with D_2O, NH); amino acid analysis (after acidic hydrolysis): 2d 0.95, Gly 1.97, Phe 1.00, and Leu 1.01.

Anal.—Calc. for $C_{30}H_{39}N_5O_6$·H_2O: C, 61.7; H, 7.1; N, 12.0. Found: C, 61.7; H, 6.8; N, 11.6.

[(2-Aminotetralyl)carbonyl] glycylglycyl -L- phenylalanyl -L- leucine Methyl Ester (IIIe)—Compound IIIe was prepared as above from IIId in 11% yield, mp 165–170°C. TLC (silica, chloroform–methanol–acetic acid, 120:90:5): R_f 0.61; IR (KCl): 1740, 1690, 1678, 1655, 1550, and 1522 cm^{-1}; ^{1}H-NMR ($CDCl_3$): δ 0.92 [m, 6, leucine δ-$(CH_3)_2$], 1.39 (m, 2, leucine β-CH_2), 1.58 (m, 2, tetralin 3-H), 1.82 (br s, 2, exchangeable with D_2O, NH_2), 1.92 (m, 1, leucine γ-CH), 3.01 (m, 4, tetralin 1- and 4-H), 3.38 (m, 2, phenylalanine β-CH_2), 3.70 and 3.72 (2 × s, 3, leucine OCH_3), 3.98 (m, 2, one of glycine α-CH_2's), 4.05 (m, 2, one of glycine α-CH_2's), 4.46 (m, 1, leucine α-CH), 4.82 (m, 1, phenylalanine α-CH), 6.88 (m, 1, exchangeable with D_2O, NH), 6.90–7.45 (m, 9, ArH), 7.54 (m, 1, exchangeable with D_2O, NH), 7.64 (m, 1, exchangeable with D_2O, NH), and 8.64 ppm (m, 1, exchangeable with D_2O, NH); amino acid analysis (after acidic hydrolysis): 3d 0.92, Gly 2.02, Phe 1.00, and Leu 1.12.

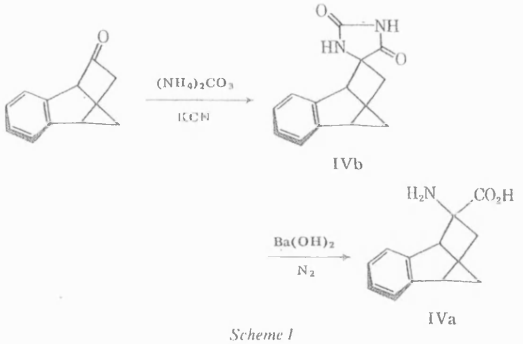
Anal.—Calc. for $C_{31}H_{41}N_5O_6$·H_2O: C, 62.3; H, 7.2; N, 11.7. Found: C, 62.8; H, 6.7; N, 11.2.

[(endo-2-Aminobenzonorbornenyl)carbonyl] glycylglycyl -L- phenylalanyl-L-leucine Methyl Ester (IVe)—This compound was prepared as above from IVd in 31% yield, mp 113–116°C. TLC (silica, chloroform–methanol–acetic acid, 120:90:5): R_f 0.78; IR (KCl): 1742, 1703, 1688, 1657, 1560, 1545, and 1520 cm^{-1}; ^{1}H-NMR ($CDCl_3$): δ 0.85 [m, 6, leucine δ-$(CH_3)_2$], 1.32 (m, 2, leucine β-CH_2), 1.52 (d of d, 1, benzonorbornene C-3 *endo*-H), 1.60 (m, 1, leucine γ-CH), 1.70 (m, 2, benzonorbornene C-9 H), 1.92 (m, 2, benzonorbornene C-3 *exo*-H), 2.47 (m, 2, benzonorbornene 1- and 4-H), 3.08 (m, 2, phenylalanine β-CH_2), 3.34 (br s, 2, exchangeable with D_2O, NH_2), 3.65 (m, 3, leucine OCH_3), 3.90 (m, 2, one of glycine α-CH_2's), 4.04 (m, 2, one of glycine α-CH_2's), 4.52 (m, 1, leucine α-CH), 4.76 (m, 1, phenylalanine α-CH), 6.80–7.56 (m, 9, ArH), 7.02 (m, 1, exchangeable with D_2O, NH), 7.63 (m, 1, exchangeable with D_2O, NH), 7.77 (m, 1, exchangeable with D_2O, NH), and 8.70 ppm (m, 1, exchangeable with D_2O, NH); amino acid analysis (after acidic hydrolysis): 4d 0.91, Gly 2.4, Phe 1.00, and Leu 1.01.

Anal.—Calc. for $C_{32}H_{41}N_5O_6$·H_2O: C, 63.1; H, 7.1; N, 11.5. Found: C, 62.9; H, 7.4; N, 11.5.

Pharmacology—Compounds were evaluated for analgesic properties in albino mice (Tuck, TFW strain) by the following procedures. *In vitro* testing was carried out by measuring the inhibition of electronically stimulated contractions of the guinea pig ileum myenteric plexus muscle using the method of Kosterlitz and Watt (38) and by measuring the inhibition of mouse vas deferens tissue after stimulation with twin rectilinear pulses 10 ms apart (39). *In vivo* evaluation was carried out using the mouse hot-plate test (40).

[13] Model EF03, Edwards.



Scheme I

RESULTS

Chemistry—The amino acids IIa and IIIa were prepared by literature methods (34, 35). Synthetic routes to IVa and Va were designed based on the stereochemistry of the products that have been obtained from Strecker and Bucherer reactions on norbornane-2-one and related compounds (41–47). Reaction of benzonorbornen-2-one with ammonium carbonate and KCN afforded exclusively the expected spirohydantoin, IVb, which could be hydrolyzed with barium hydroxide to the *endo*-amino acid, IVa (Scheme I). The unequivocal structural assignment of IVa was determined from data obtained from ^{1}H- and ^{13}C-NMR spin-spin coupling data and from two-dimensional ^{1}H-NMR studies, both of which are reported by us elsewhere (48). Attempted synthesis of Va from benzonorbornen-2-one *via* a modified Strecker synthesis resulted in formation of only the *endo*-amino acid IVa (48).

The amino acids IIa, IIIa, and IVa were each *N*-protected with carbobenzoxy chloride in base and coupled with tetrapeptide VI (37) using the *N*,*N*′-dicyclohexylcarbodiimide-1-hydroxybenztriazole method. Hydrogenolysis of the protected pentapeptides IId, IIId, and IVd and purification of the resulting deprotected products by gradient buffer elution from carboxymethylcellulose at pH 5.1 afforded the pentapeptide methyl esters IIe, IIIe, and IVe.

Pharmacology—Compound IIe exhibited only weak analgesic properties when evaluated on electrically stimulated guinea pig ileum, showing an ID_{50} of 3.8 μm (ID_{50} values for morphine, Met enkephalin, and Leu enkephalin on the same preparation are 90.5, 86.8, and 450 nM, respectively), whereas compounds IIIe and IVe were inactive. None of the pentapeptides were active analgesics in the mouse vas deferens preparation or in the hot-plate test in mice after subcutaneous and intracerebroventricular injection.

CONCLUSIONS

This study has developed synthetic routes, based on classical solution techniques, which can be used to prepare Leu enkephalin derivatives in which the terminal tyrosine-1 residue has been replaced by a variety of conformationally restrained amino acid moieties, as represented in structures IIe, IIIe, and IVe. None of these pentapeptides possess any significant analgesic activity, both *in vitro* and *in vivo*, which is consistent with the observation that enkephalins lacking an aromatic *para*-hydroxy group in the tyrosine-1 moiety are inactive as analgesics (4, 7). The synthetic procedures developed in this study should be of value in the preparation of Leu enkephalin derivatives incorporating aromatic hydroxylated derivatives of IIa, IIIa, and IVa in place of the tyrosine-1 moiety.

REFERENCES

(1) J. A. H. Lord, A. A. Waterfield, J. Hughes, and H. W. Kosterlitz, "Opiates and Endogenous Opioid Peptides," H. W. Kosterlitz, Ed. North-Holland, Amsterdam, 1976.

(2) S. H. Snyder, *Sci. Am.*, **236**, 44 (1977).

(3) J. Morley, *Annu. Rev. Pharmacol. Toxicol.*, **20**, 81 (1980) and references cited therein.

(4) L. Terenius, A. Wahlström, G. Lindenberg, S. Karlsson, and U. Ragnarsson, *Biochem. Biophys. Res. Commun.*, **71,** 175 (1976).

(5) N. S. Agarwal, V. J. Houby, R. Katz, W. Klee, and M. Nierenberg, *Biochem. Biophys. Res. Commun.*, **76,** 129 (1977).

(6) C. R. Bedell, R. B. Clark, G. W. Hardy, L. A. Lowe, F. B. Ubatuba, J. R. Vane, S. Wilkinson, K-L. Chang, P. Cuatrecasas, and R. J. Miller, *Proc. R. Soc. London B*, **198,** 249 (1977).

(7) D. H. Coy, A. J. Kastin, A. V. Schally, O. Morin, N. G. Caron, F. Labrie, J. M. Walker, R. Fertel, G. G. Berntson, and C. A. Sandman, *Biochem. Biophys. Res. Commun.*, **73,** 632 (1976).

(8) J. S. Shaw and M. J. Turnbull, *Eur. J. Pharmacol.*, **49,** 313 (1978).

(9) S. Bajusz, A. Z. Ronai, J. I. Szekely, L. Graf, Z. Dunai-Kovacs, and I. Berzetei, *FEBS Lett.*, **76,** 91 (1977).

(10) E. T. Wei, L. F. Tseng, H. H. Loh, and C. H. Li, *Life Sci.*, **21,** 321 (1977).

(11) D. Yamashiro, L. F. Tseng, and C. H. Li, *Biochem. Biophys. Res. Commun.*, **78,** 1124 (1977).

(12) A. S. Dutta, J. J. Gormley, C. F. Hayward, J. S. Morley, J. S. Shaw, G. J. Stacey, and M. J. Turnbull, *Life Sci.*, **21,** 559 (1977).

(13) P. W. Schiller, C. F. Yam, and M. Lis, *Biochemistry*, **16,** 1831 (1977).

(14) J-K Chang, B. T. W. Fong, A. Pert, and C. B. Pert, *Life Sci.*, **18,** 1473 (1976).

(15) S. Combrisson, B. P. Roques, and R. Oberlin, *Tetrahedron Lett.*, **1976,** 3455.

(16) H. E. Bleich, J. D. Cutnell, A. R. Day, R. J. Freer, J. A. Glasel, and J. F. McKelvy, *Proc. Natl. Acad. Sci. USA*, **73,** 2589 (1976).

(17) J. L. De Coen, C. Humblett, and M. H. J. Koch, *FEBS Lett.*, **73,** 38 (1977).

(18) B. E. Maryanoff and M. J. Zelesko, *J. Pharm. Sci.*, **67,** 590 (1978).

(19) R. H. B. Galt, *J. Pharm. Pharmacol.*, **29,** 711 (1977).

(20) A. S. Horn and J. R. Rodgers, *Nature (London)*, **260,** 795 (1976).

(21) W. A. Klis, E. Nawrocka, T. Z. Siemon, A. P. Plech, Z. Stachura, and Z. S. Herman, *Biorg. Chem.*, **8,** 255 (1979).

(22) F. A. Gorin and G. R. Marshall, *Proc. Natl. Acad. Sci. USA*, **74,** 5179 (1977).

(23) B. P. Roques, C. Garby-Jaureuiberry, and R. Oberlin, *Nature (London)*, **262,** 778 (1976).

(24) C. R. Jones, W. A. Gibbons, and V. Garskey, *Nature (London)*, **262,** 779 (1976).

(25) E. R. Stimson, Y. C. Meinwald, and H. A. Scheraga, *Biochemistry*, **18,** 1661 (1979) and references cited therein.

(26) J. DiMaio, P. W. Schiller, and B. Belleau, "Proceedings American Peptide Symposium," Vol. 6, E. Gross and J. Meienhofer, Eds., Pierce Chem. Co., Rockford, Ill., 1979, p. 889.

(27) M. C. Summers and R. J. Hayes, *FEBS Lett.*, **113,** 99 (1980).

(28) D. Hudson, R. Sharpe, and M. Szelke, *Int. J. Pept. Protein Res.*, **15,** 122 (1980).

(29) B. P. Roques, G. Gacel, M.-C. Fournie-Zaluski, B. Senault, and J.-M. Lecomte, *Eur. J. Pharmacol.*, **69,** 109 (1979).

(30) J. DiMaio and P. W. Schiller, *Proc. Natl. Acad. Sci. USA*, **77,** 7162 (1980).

(31) G. D. Smith and J. F. Griffin, *Science*, **199,** 1214 (1978).

(32) T. L. Blundell, L. Hearn, I. J. Tickle, R. A. Palmer, B. A. Morgan, G. D. Smith, and J. F. Griffin, *Science*, **205,** 220 (1979).

(33) D. M. Rice, R. J. Wittebort, R. G. Griffin, E. Meirovitch, E. R. Stimson, Y. C. Meinwald, J. H. Freed, and H. A. Scheraga, *J. Am. Chem. Soc.*, **103,** 7707 (1981).

(34) H. Fletcher, P. B. Russel, and H. E. Album, *U.S. Pat.*, 3,532,744; 1970; *Chem. Abstr.*, **74,** P42218k (1971).

(35) R. M. Render, B. H. Butcher, D. A. Buxton, and D. J. Howells, *J. Med. Chem.*, **14,** 892 (1971).

(36) D. J. Sandman, K. Mislow, W. P. Giddings, J. Dirlam, and G. C. Hanson, *J. Am. Chem. Soc.*, **90,** 4877 (1968).

(37) E. Vilkas, M. Vilkas, and J. Sainton, *Int. J. Pept. Protein Res.*, **15,** 29 (1980).

(38) H. W. Kosterlitz and A. J. Watt, *Br. J. Pharmacol. Chemother.*, **33,** 266 (1963).

(39) J. Hughes, H. W. Kosterlitz, and F. M. Leslie, *Br. J. Pharmacol.*, **53,** 371 (1975).

(40) N. B. Eddy and D. Leimbach, *J. Pharmacol. Exp. Ther.*, **107,** 385 (1953).

(41) S. N. Rastogi, J. S. Bindra, and N. Anand, *Indian J. Chem.*, **9,** 1175 (1971).

(42) Y. Maki, T. Masugi, and K. Ozeki, *Chem. Pharm. Bull.*, **21,** 2466 (1973).

(43) H. S. Trager and H. N. Christensen, *J. Am. Chem. Soc.*, **94,** 968 (1972).

(44) Y. Maki and T. Masugi, *Chem. Pharm. Bull.*, **21,** 685 (1973).

(45) Y. Maki, M. Sato, and K. Obata, *Chem. Pharm. Bull.*, **13,** 1377 (1965).

(46) G. G. Trigo, C. Avendano, S. Emila, J. T. Edward, and S. Wong, *Can. J. Chem.*, **57,** 1456 (1979).

(47) G. L. Grunewald, S. H. Kuttab, M. A. Pleiss, J. B. Mangold, and P. Soine, *J. Med. Chem.*, **23,** 754 (1980).

(48) W. J. Layton, and S. L. Smith, P. A. Crooks, T. Decks, and R. D. Waigh, *J. Chem. Soc. Perk. Trans. I*, in press.

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THE FIRST PROTOBERBERINE ALKALOID ANALOGUE WITH *IN VIVO* ANTIMALARIAL ACTIVITY

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Abstract
2,3,11,12-Tetramethoxyberbinium chloride at very low doses causes a marked reduction of parasitaemia produced by *Plasmodium chabaudi* infection in mice. The 2,3,10,11 and 13-amino-2,3,10,11-tetramethoxy analogues are inactive in this test system at the same dose levels, despite all three compounds having marked *in vitro* activity against *P. falciparum*. This is the first time that *in vivo* antimalarial activity of a protoberberine alkaloid analogue has been demonstrated. The parent alkaloid berberine also has activity *in vivo* against *P. chabaudi*, in contrast to its reported lack of activity against *P. berghei* in mice.

Despite persistent reports of the use of plant extracts containing protoberberine alkaloids in the folk treatment of malaria[1,2], and the demonstration of the potent antimalarial activity *in vitro* of a number of isolated compounds of this type[3], nobody has been able to demonstrate *in vivo* activity until now. The clearest and most systematic demonstration of the difference between *in vitro* and *in vivo* activity in this class of compounds was that conducted by Vennerstrom and Klayman at the Walter Reed Army Institute[4], who found *in vitro* potency against *Plasmodium falciparum* comparable to that of quinine, but a complete lack of effect in mice infected with *P. berghei*.

We have been developing a novel protoberberine synthesis, based on cyclisation of 1-cyano-2-benzyl-1,2,3,4-tetrahydroisoquinolines in anhydrous hydrogen fluoride. This method follows earlier work[5,7] on the synthesis of simpler isoquinolines and has proved to be effective, efficient and versatile, with yields on most steps in excess of 80%. A noted feature of this route is the intermediacy of a spiro-cyclised cation[8,9] following *ipso* attack of the protonated nitrile (1) on the benzyl substituent. The rearrangement which follows can thus give rise to 10,11-dimethoxy substitution (2b), from a 3,4-dimethoxybenzyl intermediate, or an 11,12-dimethoxy protoberberine (2a) from a 2,3-dimethoxybenzyl intermediate (Scheme 1). Previous studies, as for example

those of Vennerstrom and Klayman, have concentrated on compounds with the naturally occurring 9,10-oxygenation pattern.

The synthesis which we have developed gives rise to a 13-imino derivative (2) as the first-formed product after rearrangement. This intermediate is unstable and will either eliminate NH_3 (or hydrolyse[10] and eliminate H_2O) on attempted crystallisation (Scheme 2), giving directly and very conveniently the desired end-products (3a) or (3b). If allowed to stand in alkaline solution (2b) will oxidise to give the 13-amino salt (4). All three compounds ((3a), (3b) and (4)) have been tested against *P. falciparum* K1 multi-drug resistant strain *in vitro* and have similar IC_{50} values of 0.6 - 0.8 μg/ml, compared to the value for berberine[3] of 0.36 μg/ml.

In preliminary *in vivo* experiments neither (3b) nor (4) was active at the chosen dose level of 0.25 mg/kg. against *P. chabaudi* (AS strain) in BALB/c mice. The compound in buffered saline was given as a single intravenous dose one day after intraperitoneal infection with 10^6 parasitised red blood cells. However using a similar treatment regime compound (3a) not only markedly delayed the onset of a patent parasitaemia but significantly reduced ($p < 0.025$, unpaired Student's t test) the peak parasitaemia compared with untreated control animals (Fig 1).

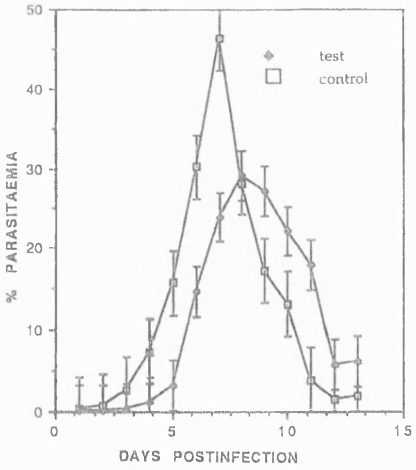


Fig 1. In vivo antimalarial activity of compound (3a) against P. chabaudi in BALB-c mice after a single i/v dose of 0.25 mg/kg: 5 mice in each group.

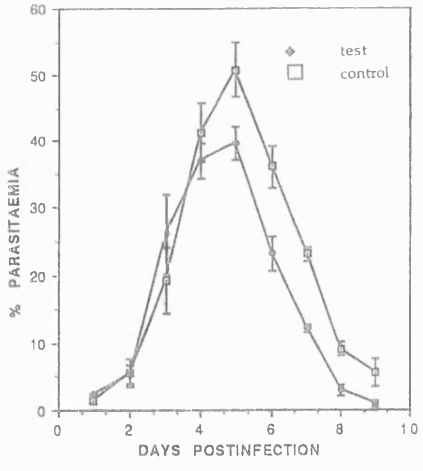
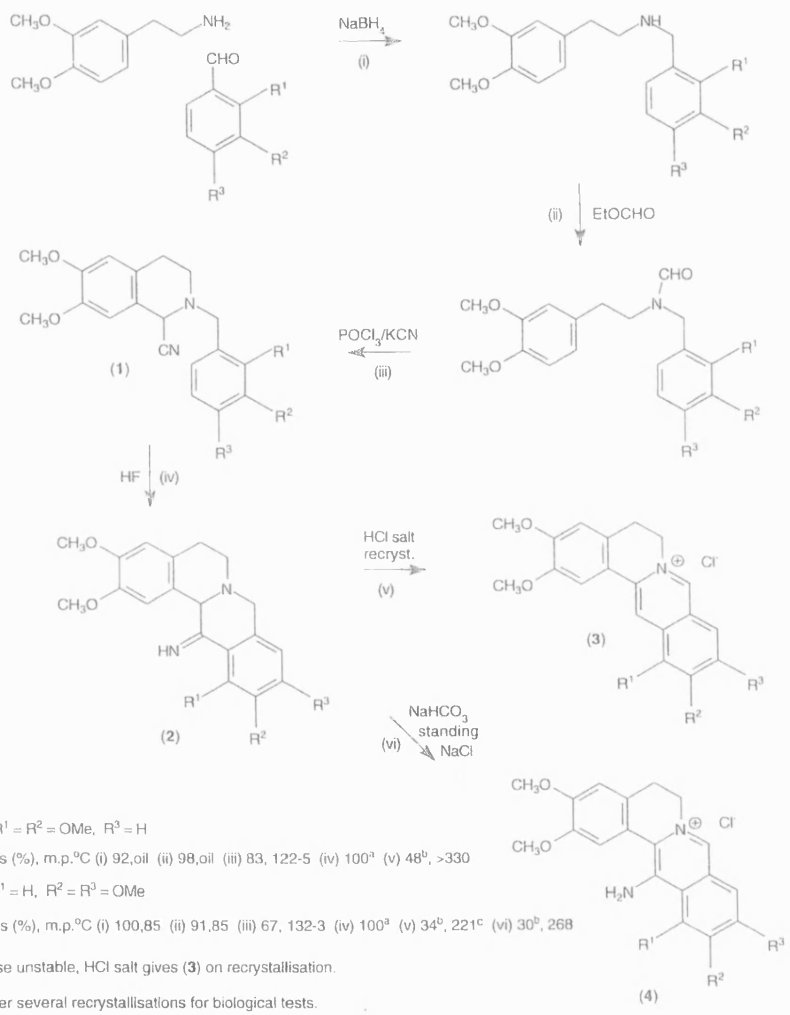


Fig 2. In vivo antimalarial activity of berberine against P. chabaudi in BALB-c mice after a single i/v dose of 0.25 mg/kg: 5 mice in each group.



(**a**) $R^1 = R^2 = OMe$, $R^3 = H$

Yields (%), m.p.°C (i) 92,oil (ii) 98,oil (iii) 83, 122-5 (iv) 100[a] (v) 48[b], >330

(**b**) $R^1 = H$, $R^2 = R^3 = OMe$

Yields (%), m.p.°C (i) 100,85 (ii) 91,85 (iii) 67, 132-3 (iv) 100[a] (v) 34[b], 221[c] (vi) 30[b], 268

[a]Base unstable, HCl salt gives (**3**) on recrystallisation.

[b]After several recrystallisations for biological tests.

[c] Lit.[11] 212-3°C from chloroform. Lit.[12] 207-8°C from methanol.

Scheme 1

This experiment has now been carried out five times, with reproducible results. In a similar experiment, berberine (0.25mg/kg) also produced a significant reduction in parasitaemia (Fig. 2).

These results show that substitution pattern is important, but may also indicate that *P. berghei* infections in mice are not an appropriate model for other *Plasmodium* infections in humans. The present results with berberine are in direct contrast to the results reported for *P.berghei* in mice[4]. It is not yet possible to predict whether differences between *in vitro* data against *P. falciparum* and *in vivo* data against *P. chabaudi* will reflect the differences for the same compounds against *P. falciparum in vitro* and *in vivo*.

Acknowledgement

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References

1. Phillipson, J.D., O'Neill, M.J. *Biologically Active Natural Products*. Hostettmann K., Lea, P.J., Eds., Clarendon: Oxford, 1987, pp 49-64.
2. Morton, J.F. Major Medicinal Plants: *Botany, Culture and Uses*. Charles C. Thomas, Pub: Springfield, USA, 1977.
3. Partridge, S.J., Russell, P.F., Anderson, M.M., Wright, C.W., Phillipson, J.D., Kirby, G.C., Warhurst, D.C., Schiff, P.L.Jr. *J. Pharm. Pharmacol. (Suppl.)*, **1990**, 42(2), 97P.
4. Vennerstrom, J.L., Klayman, D.L. *J. Med. Chem.*, **1988**, 31, 1084-87.
5. Euerby, M.R., Waigh, R.D. *J. Chem. Res*. **1987**, 36-37.
6. Euerby, M.R., Waigh, R.D. *J. Chem. Res.* **1987**, 38-39.
7. Euerby, M.R., Gavin, J.P., Olugbade, T.A., Patel, S.S., Waigh, R.D. *J. Chem. Res.*, **1991**, 58-59.
8. Euerby, M.R., Waigh, R.D. *J. Chem. Soc. Perkin Trans. 1*. **1980**, 1329-38.
9. Gavin, J.P., Waigh, R.D. *J. Chem. Soc. Perkin Trans. 1*. **1990**, 503-508.
10. Livingstone, D.A., Waigh, R.D. *J. Chem. Soc. Chem. Comm.* **1978**, 1026-27.
11. Kametani, T., Katoh, Y., Fukumoto, K. *J.Chem.Soc. Perkin Trans. 1*, **1974**, 1712-14.
12. Patra, A., Montgomery, C.T., Freyer, A.J. Guinaudeau, H., Shamma, M., Tantisewie, B., Pharadai, K. *Phytochemistry*, **1987**, 26, 547-9.

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Synthetic Benzo[c]phenanthridines with Antileukaemic Activity in Mice

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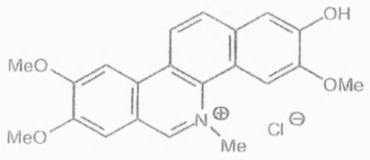
Abstract

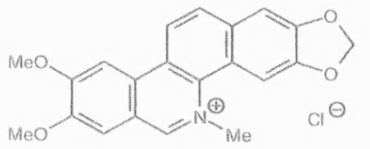
Analogues of the antitumour alkaloids fagaronine and nitidine have been synthesized to investigate their structure–activity relationships against P388 leukaemia in mice. Bulky substituents at the 12-position are well tolerated, whereas substituents larger than methoxy in the 2-position result in total loss of activity.

The effects of hydroxy groups in the 2- and 12-positions, which are respectively potency-enhancing and potency destroying, are rationalized in terms of their potential influence on bioavailability. Suitable choice of substituents may allow the production of more potent analogues with improved ability to penetrate lipid membranes.

The alkaloids fagaronine and nitidine (Fig. 1) have been known for several years (Messmer et al 1972) to possess potent antileukaemic activity in mice. More recently, they have been shown to inhibit human topoisomerases (Larsen et al 1993; Fang et al 1993), which may be the mechanism of their antileukaemic effects. We have previously described a very efficient synthesis of 2,3,8,9-oxygenated benzo[c]phenanthridines (Olugbade et al 1990) which generates a hydroxy group in the 12-position. To complete the synthesis of fagaronine requires removal of the hydroxy group, a step which can be neatly avoided by use of a vinyl synthon (Seraphin et al 1995) in place of the original ester which was a key intermediate. However, the 12-hydroxy group formed in the earlier synthesis (Olugbade et al 1990) is potentially a point of attachment of groups which might assist receptor binding, the receptor generally being assumed to be DNA in the first instance (Pezzuto et al 1983). We now describe the synthesis of quaternary benzo[c]phenanthridinium salts with 12-hydroxy and alkoxy groups (Fig. 2) and the influence of the 2- and 12-substituents on their antileukaemic activity in mice.



Fagaronine



Nitidine

FIG. 1. Structures of fagaronine and nitidine.

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Methods

The syntheses of the parent benzo[c]phenanthridines **1**, **2** and **3** were carried out as described previously (Olugbade et al 1990).

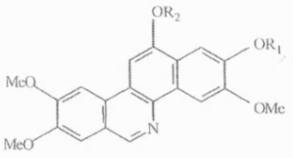
12-O-Methylation of benzo[c]phenanthridines

*2,3,8,9,12-Pentamethoxybenzo[c]phenanthridine (**4**).* The benzo[c]phenanthridine sulphate ($1.H_2SO_4$) (1 g, 2·16 mmol) was stirred in 8·4% potassium hydroxide (20 mL). Dimethyl sulphate (1 mL, 10·5 mmol) was added to the mixture and stirring continued for 20 min on a steam bath. The reaction mixture was diluted with water (20 mL) and the precipitate collected by filtration and washed thoroughly with water. The crude product (0·87 g) was purified by column chromatography on basic alumina eluting with chloroform : petroleum (b.p. 40–60°C) to give the ether **4** as a white solid (0·5 g, 61%); combustion analysis and properties are given in Tables 1, 2, 4, and 5.

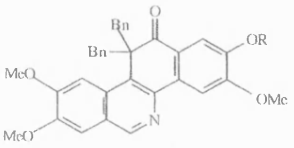
*2-Ethoxy-3,8,9,12-tetramethoxybenzo[c]phenanthridine (**5**).* Dimethyl sulphate was added (1 mL, 10·5 mmol) to a mixture of the phenol **2** (2·1 g, 5·54 mmol), and anhydrous potassium carbonate (2·2 g), in dimethylformamide (10 mL). The mixture was stirred for 24 h and an additional quantity of dimethyl sulphate (1 mL) was added. After stirring for 24 h the reaction mixture was diluted with water and extracted with chloroform. The extract was washed with water and concentrated. The residue was diluted with diethyl ether and the product **5** collected by filtration as a brown solid (1·5 g, 69%); combustion analysis and properties are given in Tables 1, 2, 4 and 5.

De-ethylation at 2-position

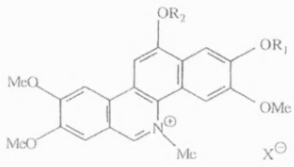
*2-Hydroxy-3,8,9,12-tetramethoxybenzo[c]phenanthridine (**6**).* The ethoxybenzo[c]phenanthridine **5** (1·4 g, 3·56 mmol) in



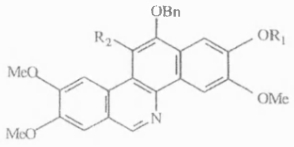
1 R_1 = Me, R_2 = H
2 R_1 = Et, R_2 = H
3 R_1 = R_2 = H
4 R_1 = R_2 = Me
5 R_1 = Et, R_2 = Me
6 R_1 = H, R_2 = Me
7 R_1 = Bn, R_2 = Me



14 R = Me
15 R = Et



16 R_1 = R_2 = Me, X = $MeSO_3$
17 R_1 = Me, R_2 = Bn, X = $MeSO_3$
18 R_1 = Et, R_2 = Bn, X = $MeSO_3$
19 R_1 = R_2 = Bn, X = SO_3
20 R_1 = Me, R_2 = H, X = Cl
21 R_1 = R_2 = H, X = Cl
22 R_1 = Bn, R_2 = Me
23 R_1 = H, R_2 = Me



8 R_1 = Me, R_2 = H
9 R_1 = Me, R_2 = Bn
10 R_1 = Et, R_2 = H
11 R_1 = Et, R_2 = Bn
12 R_1 = Bn, R_2 = H
13 R_1 = R_2 = Bn

1 ⟶ 8 + 9 + 14
2 ⟶ 10 + 11 + 15
3 ⟶ 12 + 13

FIG. 2. Structures of synthesized compounds.

concentrated sulphuric acid (98%, 12 mL) was heated at 55°C for 1 h. The cooled mixture was diluted with ice, basified with solid sodium bicarbonate and treated with a 3 : 1 mixture of chloroform and ethanol. The inorganic matter was removed by filtration. The organic filtrate was washed with water and evaporated to dryness to give **6** as a greyish-white powder (1·03 g, 77%). Combustion analysis and properties are given in Tables 1, 2, 4 and 5.

Benzylation of phenolic benzo[c]phenanthridines

Method 1. The benzo[c]phenanthridine sulphate $1.H_2SO_4$ (0·49 g, 1 mmol) was dissolved in 5% sodium hydroxide (12 mL) with warming. Benzyl bromide (0·5 mL, 4 mmol) was added and the mixture heated at 110°C for 1 h. The cooled mixture was extracted with chloroform and concentrated. The residue was treated with diethyl ether and the precipitate collected by filtration. The crude product was recrystallized from a mixture of chloroform and petroleum (b.p. 60–80°C) to give the geminal dibenzylbenzo[c]phenanthridine **14** (0·35 g, 60%). Combustion analysis and the properties of the ketone are summarized in Tables 1, 2, 4 and 5.

Method 2 (General). A mixture of phenolic benzo[c]phenanthridine (3 mmol), anhydrous potassium carbonate (7 mmol), dimethylformamide (15 mL) and benzylbromide (0·5 mL, 4 mmol) was stirred for 1 h. An additional amount of benzyl bromide (0·5 mL, 4 mmol) was added and stirring continued for 90 min. For the diphenol **3**, twice these molar proportions of potassium carbonate and benzyl bromide were used. Finally the reaction mixture was diluted with 5% sodium hydroxide (20 mL). The product was extracted with chloroform or collected by filtration. Crystallization from chloroform-petroleum (b.p. 60–80°C) gave the major component. The mother liquor was evaporated and the residue chromatographed on a column of alumina eluting with petroleum (b.p. 60–80°C) – chloroform mixtures to give the minor components. The combustion analysis, yields, properties and ^1H NMR spectral data are provided in Tables 1, 2, 4 and 5.

Quaternization of benzo[c]phenanthridines

Method 1. General procedure. A mixture of the dry benzo[c]phenanthridine base (2 mmol) and fresh methyl methanesulphonate (4 mL) was refluxed at 170–180°C under anhydrous conditions for 20 min. The reaction mixture was allowed to cool and then diluted with dry diethyl ether. The resulting precipitate was collected by filtration, redissolved in chloroform or chloroform-ethanol mixture and precipitated with diethyl ether. The final precipitate was collected by filtration and washed thoroughly with diethyl ether. The combustion analysis, yields and properties of the quaternary products thus obtained are provided in Tables 2, 3, 4 and 5.

Method 2. General procedure. A mixture of the dry benzo[c]phenanthridine base (2 mmol), methyl methanesulphonate (4 mL) and diisopropylethylamine (1 mL) was refluxed at 170–180°C under anhydrous conditions for 30 min. The reaction mixture was allowed to cool and then diluted with dry diethyl ether. The clear supernatant was carefully pipetted out. The lower resinous material was treated with a few drops of ethanol and rubbed with a spatula until a solid precipitate was

Table 1. Combustion analysis.

Compound	Found	Required	Formula
5	C, 68·3; H, 5·8; N, 3·5	C, 68·0; H, 5·7; N, 3·6	$C_{22}H_{21}NO5{\cdot}1{\cdot}5H_2O$
6	C, 67.1; H, 5.2; N, 3.4	C, 67.4; H, 5.4; N, 3.4	$C_{21}H_{19}NO_5{\cdot}0.5H_2O$
7	C, 72.3; H, 5.4; N, 2.5	C, 72.4; H, 5.6; N, 3.0	$C_{28}H_{25}NO_5{\cdot}0.5H_2O$
8	C, 72.5; H, 5.5; N, 2.85	C, 72.4; H, 5.6; N, 3.0	$C_{28}H_{25}NO_5{\cdot}0.5H_2O$
9	C, 76.3; H, 5.7; N, 2.3	C, 75.8; H, 5.8; N, 2.5	$C_{35}H_{31}NO_5{\cdot}0.5H_2O$
10	C, 7.17; H, 5.7; N, 2.5	C, 71.4; H, 6.0; N, 2.9	$C_{29}H_{27}NO_5{\cdot}H_2O$
11	C, 76.3; H, 5.8; N, 2.5	C, 76.0; H, 6.0; N, 2.5	$C_{36}H_{33}NO_5{\cdot}0.5H_2O$
12	C, 75.7; H, 5.4; N, 2.6	C, 75.5; H, 5.6; N, 2.6	$C_{34}H_{29}NO_5{\cdot}0.5H_2O$
13	C, 79.1; H, 5.9; N, 2.0	C, 79.2; H, 5.7; N, 2.3	$C_{41}H_{35}NO_5$
14	C, 75.9; H, 5.8; N, 2.3	C, 75.8; H, 5.8; N, 2.5	$C_{35}H_{31}NO_5{\cdot}0.5H_2O$
15	M+, 559.2356	559.2359	
16	C, 57.6; H, 5.5; N, 2.6	C, 57.8; H, 5.7; N, 2.8	$C_{24}H_{27}NO_8S{\cdot}0.5H_2O$
17	C, 62,5; H, 6.1; N, 2.2	C, 62.7; H, 5.6; N, 2.4	$C_{30}H_{31}NO_8S{\cdot}0.5H_2O$[a]
18	C, 61.9; H, 5.7; N, 2.1	C, 62.3; H, 5.9; N, 2.3	$C_{31}H_{33}NO_8S{\cdot}H_2O$
19	C, 65.8; H, 5.4; N, 2.0	C, 65.5; H, 5.7; N, 2.1	$C_{36}H_{33}NO_8S{\cdot}H_2O$
20	C, 60.8; H, 5.5; N, 3.0	C, 60.9; H, 5.6; N, 3.2	$C_{22}H_{22}NO_5Cl{\cdot}H_2O$
21	C, 62.3; H, 5.1; N, 3.3	C, 62.8; H, 5.0; N, 3.5	$C_{21}H_{20}NO_5Cl$[a]
22	C, 62.6; H, 5.5; N, 2.3	C, 62.7; H, 5.6; N, 2.4	$C_{22}H_{22}NO_5Cl{\cdot}H_2O$

[a]Hygroscopic (gained weight on balance).

Table 2. Yields and melting points for benzo[c]phenanthridine bases.

Compound	Yield (%)	m.p. (°C)
4	61	274–276 (dec.)[a]
5	69	240–244[d]
6	77	238–244[b]
7	72	249–256[b]
8	53	210–212[b]
9	5[c]	188–190[c]
10	70	225–227[c]
11	7[c]	194–196[c]
12	55	237–239[c]
13	10[c]	214–216[b]
14	1[c]	274–275[c]
15	1[c]	281–284[c]

[a]From diethylene glycol. [b]From $CHCl_3$-ethanol. [c]From $CHCl_3$-petroleum (b.p. 60–80°C). [d]From $CHCl_3$-methanol. [e]By-product from Method 2. 14 obtained in 60% yield by Method 1.

formed. The material was then diluted with diethyl ether, collected by filtration, and finally recrystallized twice from chloroform or chloroform-diethyl ether. The combustion analysis, yields and properties of the salts so obtained are provided in Tables 2, 3 4 and 5.

Dealkylation of quaternary benzo[c]phenanthridines

General method. A mixture of benzo[c]phenanthridine salt, glacial acetic acid (10 mL), and concentrated hydrochloric acid (5 mL) was refluxed at 70–80°C for 2 h during which a yellow precipitate was formed. The reaction mixture was filtered while still warm and the collected precipitate washed thoroughly with diethyl ether.

The combustion analysis and properties of **20** and **21** obtained by dealkylation as chloride salts are described in Tables 2, 3, 4 and 5.

Antileukaemic tests

The test system employed in the screen coded 3PS31, consisted of the ascitic fluid of lymphocytic leukaemia P388 implanted in mouse strains coded BDF, or CDF_1. Tests were conducted by the National Cancer Institute, Bethesda, Maryland, USA. The inoculum site was intraperitoneal and the

Table 3. Properties of quaternary benzo[c]phenanthridines.

Compound	Yield[a]	m.p. (°C)	Solvent	λ_{max}(E)
16	96[b]	277–281	MeOH-$CHCl_3$	234 (24,400) 283 (607,00) 316 sh (31,000) 330 sh (26,200) 410 (8,300)
17	99[c]	246–250	EtOH	235 (12,300) 283 (31,400) 317 sh (19,700) 330 sh (14,400) 410 (7,200)
18	89[b]	243–246	EtOH	237 (24,500) 285 (56,000) 320 sh (34,200) 332 sh (24,500) 412 (12,500)
19	99[c]	261–263	EtOH-$CHCl_3$	236 (23,800) 284 (57,600) 318 (39,000) 333 sh (27,200) 412 (15,200)
20	67	> 310	MeOH	236 (16,200) 287 (37,200) 320 (17,300) 333 sh (11,600) 416 (5,800) shifted to 234 (17,700) 290 (33,600) 360 (7,200) 470 (3,600) on adding NaOH
21	68	236–239	MeOH-$CHCl_3$	238 (19,800) 288 (45,800) 322 sh (27,500) 335 sh (20,100) 420 (12,100) shifted to 269 (25,300) 313 (42,100) 354 (20,500) 500 (14,000) on adding NaOH
22	96[c]	235–238	EtOH-$CHCl_3$	237 (26,200) 286 (61,800) 318 (44,500) 333 sh (29,800) 412 (17,300)

[a]From final step. [b]Quaternization by method 1. [c]Quaternization by method 2.

Table 4. Mass spectrometry data for benzo[c]phenanthridines.

Compound	m/z (%)
4	379 (M^+, 100), 364 (3.8), 336 (10)
5	393 (M^+, 100), 378 (12), 3643 (27)
6	365 (M^+, 100), 364 (17.6), 350 (11.4), 336 (3.2), 322 (12.6), 307 (13.1),306 (1.1)
7	455 (M^+, 43.4), 364 (100), 336 (10.5), 91 (20.3)
8	455 (M^+, 38.7), 364 (100), 336 (29.8), 91 (17.1)
9	545 (M^+, 7.8), 454 (100), 426 (7.0), 364 (17.0), 336 (4.8), 91 (25.8)
10	469 (M^+, 44), 378 (100), 350 (17.6), 91 (18.7)
11	559 (M^+, 6.4), 468 (100), 440 (9.9), 378 (15.3), 350 (4.0), 91 (26.7)
12	531 (M^+, 19.4), 440 (27.1), 350 (8.0), 91 (100)
13	621 (M^+, 6.2), 530 (53.8), 440 (40.0), 91 (100)
14	545 (M^+, 12.0), 454 (100), 426 (9.4), 364 (2.6), 336 (2.0), 91 (27.0)
15	559 (M^+, 23.3), 468 (100), 440 (9.8), 378 (23.4), 350 (5.2), 91 (49.6)
16	379 (100), 364 (12.6), 348 (5.6), 336 (20.6)
17	455 (50.6), 364 (100.0), 336 (40.1), 91 (29.6)
18	469 (33.0), 378 (100), 350 (50.3)
19	531 (25.9), 440 (32.0), 351 (43.3), 350 (23.0), 91 (100)
20	380 (14.6), 379 (55.2), 365 (100.0), 364 (42.3), 350 (17.1), 336 (25.9)
21	380 (4.4), 379 (14.6), 366 (20.3), 365 (88.2), 364 (26.2), 351 (100.0), 350 (52.0), 336 (15.4), 322 (22.5)

parameter for evaluation was the median survival time, (MST) defined as the median day of death for a test or control group and it was calculated according to the following formula (Geran et al 1972):

$$MST = L + (C \times j/f_M) \quad (1)$$

where L is the lower boundary of class containing median animal, and is equal to $D_M - 0{\cdot}5$, D_M is that day when total deaths are equal to or greater than A, A is (initial count + 1)/2, C is the class interval (equal to the 1 day), j is the number of deaths needed to reach medium animal from the lower class boundary and is equal to A minus total deaths prior to day D_M, f_M is the frequency of class (total deaths on day D_M. T/C (percent) was defined as the ratio of the median survival time of the test group to the median survival time of the control group expressed as a percentage. In general, a minimal increase in survival of treated animals over controls resulting in a T/C value greater than or equal to 125% was considered necessary to demonstrate activity. A reproduced T/C value greater than or equal to 125% was considered worthy of further study.

The antileukaemic test data are presented in Table 6.

Toxicity

A test material was considered toxic (Geran et al 1972) if any one of the following conditions was met in an otherwise inactive test: 34% deaths by the toxicity day (day 5); T/C greater than or equal to 85%; a negative average animal weight change difference (test minus control) greater than or equal to 4 g by the toxicity day.

Table 5. ^{1}H NMR spectra of benzo[c]phenanthridines. Unless stated otherwise, spectra were obtained in $CDCl_3$.

Compound	OCH_2CH_3	OCH_3	OCH_2CH_3	Ring CH	$C\text{-}(CH_2Ph)_2$	$O\text{-}CH_2Ph$	$ArCH_2Ph$	OH
4		4.09 × 2,4.18,4.20,4.21		7.38,7.48,7.70,7.78,8.73,9.15				
5	1.60,t,7Hz	4.08,4.14,4.15,4.19	4.33,q,7Hz	7.33,7.38,7.68,7.68,8.70,9.11				
6		4.10,4.20 × 3		7.37,7.43,7.73,7.80,8.70,9.13				
7		4.10,4.20 × 3		7,3-7.7,8H,m;7.80,1H,S; 8.73,1H,S;9.13				
8		4.08 × 2,4.13,4.20		7.3-7.7,8H,m;7.77,8.72,9.11		5.47		1.60
9		3.30,3.92,4.07,4.23		7.20-7.50,11H,m; 7.51,7.89,8.87,9.20		5.00	4.94	1.65
10	1.59,t,7Hz	4.09,4.14,4.20	4.31,q,7Hz	7.3-7.7,8H,m;7.80,8.72,9.13		5.48		
11	1.50,t,7Hz	3.30,4.07,4.22	4.13,q,7Hz	7.20-7.50 11H,m; 7.51,7.89,8.87,9.20		5.00	4.94	1.65
12		4.07,4.12,4.18		7.3-7.6,13H,m; 7.80,8.71,9.08		5.33;5.37		
13		3.20,3.96,4.18		7.13-7.44,17H,m; 7.72,8.82,9.09		4.79;5.16	4.67	
14		3.96x2,4.10,4.21		6.52br,4H,d,7Hz; 6.7-6.85,6H,m; 7.27,7.46,7.97,8.13,8.92	3.96,4.08,4H, ABq,13Hz			
15	1.50,t,7Hz	3.97,4.12,4.22	4.20,q,7Hz	6.49br,4H,d,7Hz; 6.7-6.85,6H,m; 7.27,7.43,7.92,8.10,8.92	3.96,4.10,4H, ABq,13Hz			1.60
16		4.20x3,4.34x2		7.75br,2H,S;8.12br,3H;9.28				
17		3.85,3.98,4.08,4.45		7.42-7.88,10H,m;10.10				
18	1.58,t,7Hz	3.78,3.96,4.41	4.25,q,7Hz	7.38-7.86,10H,m;10.04		5.82		
19[c]		3.86,4.00,4.40		7.38-7.60,H,m; 7.68,7.94,7.99,8.02,9.94		5.37,2H; 5.56,2H		
20[b]		4.58 × 3,4.70		8.05,8.40,8.48br,9.52, 8.40,8,.48,9.52				
21[a]		4.04,4.08,4.19		7.77,7.81,7.86,7.88,8.11,9.64		10.52, 11.40		
22		3.71,4.08,4.50,4.52		7.46-7.62,6H,m; 7.70,7.85,7.92,8.10,10.08		5.34		

[a]DMSO-d6, [b]trifluoroacetic acid, [c]CD_3OD-$CDCl_3$

Table 6. Leukaemia screen (3PS31) test results (T/C %).

Compound	Dose (mg kg^{-1})							Status
	3.12	6.25	12.5	25	50	100	200	
1[a]	98	106	96	ND	Toxic	Toxic	Toxic	Inactive
16	ND	ND	118	125	132	127	ND	Active
17	ND	124	128	139	Toxic	Toxic	Toxic	Active
18	118	112	91	ND	Toxic	Toxic	Toxic	Inactive
19	100	100	98	ND	Toxic	Toxic	Toxic	Inactive
20	ND	ND	ND	ND	99	97	99	Inactive
21	ND	ND	ND	152	153	163	156	Active

[a]As sulphate.

Discussion

Chemistry

Although 12-*O*-methylation proceeded straightforwardly to give **4** – **7**, the analogous benzylation was very sensitive to the reaction conditions in its regioselectivity. In aqueous alkali the major product was the 11,11-dibenzyl ketone **14**, formed by preferential C-alkylation. In contrast, use of dimethyl formamide gave mainly the desired 12-*O*-benzyl adduct with small quantities of the 11,12 (**9** and **11**) and 11,11-dibenzyl derivatives (**14** and **15**). The structures could readily be distinguished by IR and NMR analysis, since the 12-*O*-benzyl compounds (**8**, **10** and **12**) retained the aromatic H_{11} in the ^1H NMR spectrum and the 11,11-dibenzyl compounds (**14** and **15**) showed a C=O stretch in the IR spectrum. In support of this assignment, the benzyl methylene groups of **14** appeared as a four-proton AB quartet, centred at 4·02 ppm, while the benzyl methylene groups of **9** appeared as singlets at 4·94 and 5·00 ppm.

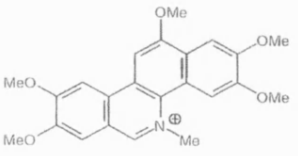
Benzylation of ellagic acid provides a precedent for these observations (Jurd et al 1959), giving mixtures of C,C and O,C dibenzylated products when treated with benzyl chloride in a pyridine/sodium hydroxide mixture. At first sight, our observations appear to indicate a change of mechanism, perhaps from S_N2 to S_N1, depending on the solvent, with *O*-alkylation preferred under S_N2 conditions. That this explanation is oversimplified is indicated from results with alkenyl halides of varying chain length (Sharples 1994).

Quaternization was expected to be relatively difficult, requiring high temperatures and concentrated reagents; the problem was overcome by the use of pure methyl methanesulphonate, which has a high boiling point. The production of methanesulphonate salts also had the useful property of increasing the water solubility for biological testing. A feature was the tendency for the quaternizing agent to hydrolyse on storage, resulting in protonation rather than quaternization of the base. The proton salts were very difficult to separate from the desired quaternary salts, a problem which was overcome by use of the non-nucleophilic base di-isopropylethylamine in the reaction mixture, giving clean products which were easy to purify.

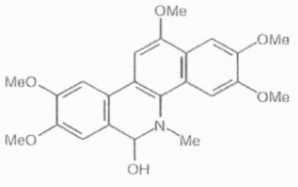
An unexpected aspect of the chemistry of this series was the very ready demethylation at position 12 in the quaternary salt **22** but not in the tertiary bases, observed when an attempt was made to prepare the 2-hydroxy-3,8,9,12-tetramethoxy analogue **23**, which for the reasons given below was expected to have greater antileukaemic activity than the compounds tested. The proposed route started with **2** and proceeded satisfactorily via 12-*O*-methylation, 2-*O*-de-ethylation in concentrated sulphuric acid and 2-*O*-benzylation to give **22**. Treatment of **22** with acid, however, gave the diphenol **21** rather than the desired 12-*O*-methyl derivative **23**. There was insufficient time in this study to attempt alternative methods for debenzylation of **22**.

Antileukaemic effects

As expected (Zee-Cheng & Cheng 1975) the tertiary base **1** was inactive. The 12-*O*-methyl quaternary salt **16** and the 12-*O*-benzyl *N*-methyl analogue **17** salts both exceeded the threshold criterion to be deemed worthy of further investigation (Geran et al 1972). The 12-*O*-benzyl derivative is particularly significant since it indicates that the binding site can accept bulky groups in this region, pointing the way for the synthesis of further analogues. In contrast, the lack of activity of the 2-ethoxy **18** and 2-benzyloxy **19** derivatives indicates a very low steric tolerance in the binding site at this point. Such an observation is in accord with the relative potencies of the alkaloid fagaronine and the synthetic derivative *O*-methylfagaronine (Zee-Cheng & Cheng 1975), the methyl group at

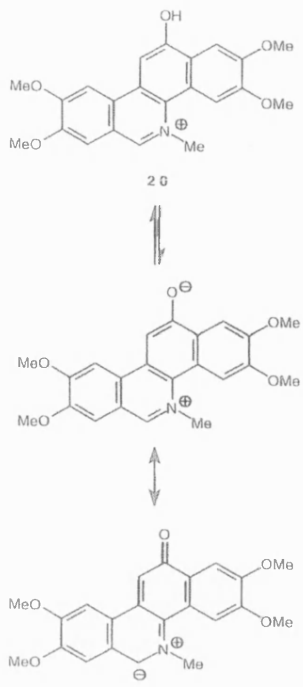


16



24

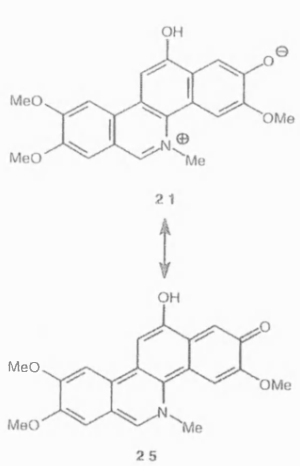
Scheme 1



Scheme 2

position 2 resulting in a substantial loss of antileukaemic activity.

The lack of activity of the 12-hydroxy analogue **20** cannot be readily attributed to poor receptor binding given the very



Scheme 3

marked activity of the 2,12-dihydroxy analogue **21**. The latter shows that this series is well worth further investigation and may indicate that penetration of cell membranes is an important determinant of potency. Most of the quaternary salts described in this study would be expected (Mitscher et al 1978; Laola & Stermitz 1979) to exist at physiological pH in a tautomeric mixture with the pseudobase form (e.g. **16** and **24** (Scheme 1)). The uncharged pseudobase would be expected to penetrate cell membranes more readily. It is possible that the 12-hydroxy group of **20** may hinder nucleophilic attack at position 6 (Scheme 2). This effect may be compensated in **21** by loss of the 2-hydroxy proton followed by quinone-like resonance to give **25** (Scheme 3).

Given the DNA-binding properties and established inhibitory effects on human topoisomerase of fagaronine, it is very likely that the antileukaemic effects of the analogues **16**–**21** are mediated first through DNA binding and then through formation of an enzyme complex. Computer modelling of the drug-DNA complex may help to clarify the steric tolerance at position 12 in this series and provide some guidance for the synthesis of yet more potent analogues.

Acknowledgements

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References

Fang, S. D., Wang, L.-K., Hecht, S. M. (1993) Inhibitors of DNA topoisomerase I isolated from the roots of *Zanthoxylum nitidum*. J. Org. Chem. 58: 5025–5027

Geran, R. I., Greenberg, N. H., Macdonald, M. M., Schumacher, A. M., Abbott, B. J. (1972) Protocol for screening chemical agents and natural products against animal tumours and other biological systems. Cancer Chemotherapy Reports 3: 1–103

Jurd, L., Palmer, K. J., Stitt, F., Shoolery, J. N. (1959) Plant polyphenols. IX: structure of the yellow product from the benzylation of ellagic acid. J. Amer. Chem. Soc. 81: 4620–4623

Laola, M. A., Stermitz, F. R. (1979) Benzophenanthridine salt equilibria. Heterocycles 12: 11–15

Larsen, A. K., Grondard, L., Couprie, J., Desoize, B., Comoe, L., Jardillier, J.-C., Riou, J.-F. (1993). The antileukaemic alkaloid fagaronine is an inhibitor of topoisomerase I and II. Biochem. Pharmacol. 46: 1403–1412

Messmer, W., Tin Wa, M., Fong, M., Bevelle, C., Farnsworth, N., Abraham, D., Trojanek, J. (1972) Fagaronine, a new tumour inhibitor isolated from *Fagara zanthoxyloides* Lam. (Rutaceae). J. Pharm. Sci. 61: 1858–1859

Mitscher, L. A., Park, Y. H., Clark, D., Clark, G.W., Hammesfahr, P. D., Wu, W. N., Beal, J. (1978) Antimicrobial agents from higher plants. An investigation of *Hunnemannia fumariaefolia* pseudoalcoholates of sanguinarine and chelerythrine. Lloydia 41: 145–150

Olugbade, T. A., Waigh, R. D., Mackay, S. P. (1990) A very short route to fully aromatic 2,3,8,9 and 2,3,8,9,12-oxygenated benzo[c]-phenanthridines. J. Chem. Soc. Perkin Trans. 1: 2657–2660

Pezzuto, J. M., Autosiak, S. K., Messmer, W. M., Slaytor, M. B., Honig, G. R. (1983) Interaction of the antileukaemic alkaloid fagaronine and nucleic acids. Chem. Biol. Interact. 43: 323–339

Seraphin, D., Lynch, M. A., Duval, O. (1995) A simple and efficient route to benzo[c]phenanthridines. Tetrahedron Lett. 36: 5731–5734

Sharples, S. L. (1994) Synthetic approaches to benzo[c]phenanthridinyl derivatives of bronopol PhD thesis, University of Manchester

Zee-Cheng, K., Cheng, C. C. (1975) Preparation and antileukaemic activity of some alkoxybenzo[c]phenanthridium salts and corresponding dihydro derivatives. J. Med. Chem. 18: 66–71

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The Antimalarial Activity of Berberine and Some Synthetic Analogues

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Abstract

Berberine chloride and ten synthetic analogues were tested for antimalarial activity and for interaction with DNA. Antimalarial activity was assessed by measurement of inhibition of growth of *Plasmodium falciparum* in-vitro, using chloroquine-sensitive and -resistant strains. DNA interactions were assessed by UV spectrophotometric studies, thermal denaturation measurements and gel electrophoresis.

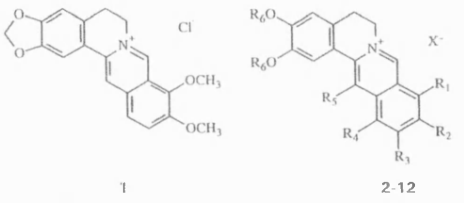
Four of the compounds including berberine chloride were found to show some interaction with DNA which showed some correlation with antimalarial activity.

Berberine is known to inhibit several enzymes, as well as DNA and these results suggest that DNA is not the only site of antimalarial action of these analogues.

Malaria is one of the most important diseases in the world, with an estimated mortality of 1–2·5 million per year, mainly among children (Cox 1991). Prospects for the control of malaria have been seriously hampered by the emergence of resistance to all available antimalarial drugs (Bruce-Chwatt 1993).

Recent work has cast doubts on the potential for vaccination in the control of falciparum malaria (d'Alessandro et al 1995), so that effective treatment remains the major control method at present, and will always be necessary for infected cases.

Protoberberine alkaloids have been suggested as candidates in the search for new antimalarials. The parent compound, berberine (**1**, Fig. 1), was reported to exhibit a potency in-vitro comparable with that of quinine (Cox 1991). It has been extensively used in folk medicine against many different diseases (Kondo 1976; Creasey 1979), and is active against the protozoan *Leishmania donovani* (Ghosh et al 1985; Vennerstrom et al 1990). The alkaloid was reported to be inactive against *Plasmodium berghei* in mice (Vennerstrom & Klayman 1988), but recent results have shown in-vivo activity against *Plasmodium chabaudi* in mice (McCall et al 1994).



1 **2-12**

FIG. 1. Chemical structures of berberine (**1**) and synthetic analogues (**2–12**). See Table 2 for explanation of the R groups.

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The mode of action of berberine is unknown, although the drug has been shown to interact with a number of dehydrogenase enzymes and with DNA (Kapp & Whiteley 1991). To test whether the DNA interaction was a determinant of antimalarial activity it was first necessary to synthesize a range of analogues. A flexible synthesis involving the hydrogen fluoride cyclization of benzylamino nitriles was developed (McCall et al 1994) supplying the protoberberine analogues (**2–12**). We now describe the effects of these analogues (Fig. 1) against *Plasmodium falciparum* in-vitro, using both chloroquine-sensitive and -resistant isolates and some physicochemical studies of the interactions of these compounds with DNA.

Materials and Methods

Materials

Berberine chloride and calf thymus DNA were obtained from Sigma Chemical Co. The other protoberberine alkaloids were prepared as described previously (McCall et al 1994). All were obtained as the chloride salt except for compound **5** which was obtained by demethylation of compound **2** using HBr, giving the bromide.

In-vitro antimalarial activity tests

The test was based on previously reported methods (Desjardins et al 1979; O'Neill et al 1985). Laboratory cultures of *P. falciparum* were maintained by standard methods. For the test, 25-μL aliquots of culture medium were added to all the wells of a 96-well flat-bottomed microculture plate (Sterilin, UK). Samples (25 μL) of test solutions were added, in duplicate, to the first wells and a Titertek motorized hand diluter (Flow Labs, UK) used to make serial 2-fold dilutions of each sample over a 64-fold concentration range. Samples (200 μL) of a 1·5% (v/v)

suspension of parasitized red blood cells in culture medium (0·4% parasitaemia, growth rate >3 per 48 h) were added to all test wells. Parasitized and non-parasitized red blood cells and solvent controls were incorporated in all tests. Plates were incubated at 37°C in a gas mixture of 3% CO_2, 5% O_2, 92% N_2. After 48 h each well was pulsed with 25 μL culture medium containing 0·5 μCi [^{3}H]hypoxanthine and incubated for a further 18 h. The contents of each well were then harvested onto glass fibre filters, washed thoroughly with distilled water, dried, and radioactivity measured by liquid scintillation. The regression function log (counts min^{-1}) = a + bx (log drug concentration) was calculated using duplicate points above and below the counts min^{-1} midpoint between the parasitized and non-parasitized controls. The concentration causing 50% inhibition of radioisotope incorporation (ID50) was determined by interpolation (Sixsmith et al 1984). This method gives a value equal to 100·4 ± 10·6% (mean ± s.d.) of the value estimated by nonlinear regression analysis (Watkins et al 1984). Statistical differences between means of small populations were examined using Student's *t*-test.

The K39 strain of *P. falciparum* was isolated from Western Kenya and is sensitive to chloroquine and sulfadoxine in-vitro but resistant to pyrimethamine. The V1/S strain was isolated in Vietnam and is resistant to chloroquine, pyrimethamine and sulfadoxine.

UV-vis spectrophotometry

UV-vis spectra and measurements were recorded using a Pye Unicam PU 8800 spectrophotometer, connected to an SPX 876 series 2 Temperature Programmer.

For optical measurements, DNA-alkaloid complexes were prepared by mixing fixed amounts of protoberberine ligand (0·25 mL 1 mM) with varying volumes of DNA (0, 0·1, 0·2, 0·5, 1·0, 1·5 mL of 1 mg mL^{-1} solution) and made up to 10 mL with Tris buffer (0·03 M, pH 7·0). This gave a DNA: ligand ratio of 0:1, 1:1, 2:1, 5:1, 10:1 and 15:1, respectively. The solutions were allowed to stand for 3 min at room temperature. For each solution the absorption spectrum was recorded (superimposed) over a wavelength range of 500–300 nm. 'DNA concentration' refers to the molar concentration of base pairs.

Thermal denaturation measurements

DNA (200 μL, 1 mg mL^{-1}) was mixed with ligand (50 μL or 1 mM) in Tris buffer, pH 7·0. The mixture was made up to 10 mL with Tris buffer and allowed to stand at room temperature for 30 min to permit equilibration. A sample (3 mL) was then pipetted into a quartz cell and absorption measurements at 260 nm were taken as the temperature of the solution was raised from 50–98°C at a rate of 0·5°C min^{-1}. T_m was estimated as the temperature at which the midpoint of the increase in absorption occurred. The procedure was repeated with no ligand present. All measurements were duplicated.

Gel electrophoresis studies

Vacuum-desiccated plasmid DNA, pGEX-2T (obtained and amplified by A. Awan), was made up to a stock solution of approximately 400 ng μL^{-1} with sodium phosphate buffer (0·1 M, pH 7·5). The protoberberine ligand was dissolved in Tris buffer (0·03 M, pH 7·0) to a concentration of 0·359 mM. Compound **3**, because of solubility problems, was dissolved in 9 parts of Tris buffer and 1 part of dimethylsulphoxide.

DNA stock solution (1 μL) was whirlmixed with appropriate volumes of the ligand stock solution (i.e. 0, 0·5, 1·0, 1·5 and 2·0 μL). The mixtures were allowed to stand for 1 h to equilibrate. Bromophenol blue solution (1 μL, 0·25% bromophenol blue, 40% (w/v) sucrose in H_2O) was added to each sample and the samples left to stand for a further 5 min, then subjected to electrophoresis for 1·5 h in horizontal submarine agarose gels (1·0% w/v, 20 × 8 × 0·6 cm) at a constant 50 V, using as gel and reservoir buffer TAE buffer (0·04 M Tris-acetate, 0·001 M EDTA, pH 8·0). On completion of electrophoresis the gels were immersed in a solution of ethidium bromide (0·5 μg mL^{-1}). After 20 min, the DNA bands were visualized by the fluorescence of the DNA-bound ethidium bromide using a mid-range UV transilluminator. Photographs of the gels were taken under UV light with Polaroid type 665 black and white positive/negative film.

Molecular modelling

This was carried out on a Silicon Graphics IRIS 4D/240 GTX hardware system using the Polygen QUANTA and CHARM software.

Results and Discussion

The first test of DNA binding to be carried out on these compounds was the UV spectrophotometric binding assay. This is the easiest and quickest way to decide which compounds show no intercalation. It is well known that the long wavelength absorption band of ligands which intercalate between the base pairs of DNA undergoes a shift to longer wavelengths (the bathochromic shift) with a corresponding decrease in absorbance on intercalation into DNA. The shift is believed to be a consequence of an overlap between the π-electrons of the nucleic acid base pairs and the bound ligand chromophore. Isosbestic points are generally observed when the UV spectra of the intercalator in the presence of increasing concentrations of DNA are overlaid. These are points at which all the spectra converge and the existence of such points implies that only a single spectroscopically distinct bound form of the drug molecule exists.

Solutions with DNA base pairs:ligand ratios varying between 0:1 and 15:1 were prepared for each of the 12 compounds, and their UV-vis spectra overlaid. Compounds **1**, **2** and **3** all gave small bathochromic shifts with isosbestic points. Compound **4**, in which C13 is substituted with an amino group, gave inconclusive results. There was some convergence of the lines but no clear isosbestic points and no bathochromic shift. The results of the UV-vis spectrometry titrations are summarized in Table 1.

The remaining compounds (**5**–**12**), in which there is substitution with bulky groups, gave rise to unchanged UV spectra on addition of DNA. These results show that this substitution inhibits binding to DNA.

On heating, DNA undergoes strand separation, the T_m (midpoint of the thermal transition profile) being detectable by monitoring the increase in absorbance at 260 nm as the

Table 1. Bathochromic shifts and isosbestic points of protoberberine alkaloids on addition of 15 equivalents of DNA and effect of selected protoberberines on the melting temperature of calf thymus DNA (11·67 μM).

Compound	γ max	Δγ (nm)	Isosbestic points (nm)	ΔT_m (°C)
1	343	2	354, 443	2·5
2	307	2	344, 362, 388, 410	3·0
3	317	2	352, 404	5·0
4	Inconclusive	Inconclusive	Not applicable	1·5
5–12	No binding	No binding	Not applicable	Not applicable

Table 2. Antimalarial activity of protoberberines against chloroquine-sensitive and -resistant strains of *Plasmodium falciparum* in-vitro.

Compound	R_1	R_2	R_3	R_4	R_5	R_6	IC50 (μM)		V1/S/K39
							K39	V1/S	
1							0·08	1·98	24·8
2	H	OCH_3	OCH_3	H	H	CH_3	0·10	2·22	22·2
3	H	H	OCH_3	OCH_3	H	CH_3	0·59	5·19	8·80
4	H	OCH_3	OCH_3	H	NH_2	CH_3	0·16	0·84	5·25
5	H	OH	OH	H	H	H	0·40	0·64	1·60
6	H	OCH_3	OCH_3	H	NHCOPh	CH_3	7·00	82·3	11·76
7	H	OCH_3	OCH_3	H	$NHCOCH_2CH_2CH_3$	CH_3	4·29	84·0	19·6
8	OCH_3	OCH_3	H	SCH_3	H	CH_3	0·35	1·59	4·54
9	H	OCH_3	OCH_3	H	$NHCOCH_3$	CH_3	0·73	3·14	4·30
10	H	OCH_3	OCH_3	H	$NHCOCH_2CH_2Cl$	CH_3	2·06	87·9	42·7
11	H	OCH_3	OCH_3	H	NHCONHBz	CH_3	0·60	8·22	13·7
12	H	OCH_3	OCH_3	H	$NHCO(CH_2)_6CH_3$	CH_3	1·28	10·8	8·44
Chloroquine							0·0125	0·178	14·2

temperature is raised from 50°C (at which all the DNA is double stranded) to 98°C (at which temperature it is all single stranded).

T_m varies according to the environment of the DNA and is about 70°C for calf thymus DNA in Tris buffer. Intercalators raise the value of T_m by hindering the internal motion of base pairs and sugars at the site of intercalation, resulting in stabilization of the DNA duplex. Intercalators with high affinity for DNA stabilize the duplex more effectively than those with lower affinity.

Compounds **1**–**4** were tested for their ability to raise the T_m for calf thymus DNA. The results are summarized in Table 1. From these data it appears that **2** binds with about the same affinity as berberine (**1**), whereas **3** has greater and **4** less affinity for calf thymus DNA. None of the increases in T_m is very large, suggesting fairly weak binding.

The effect of berberine chloride and compounds **2**, **3** and **4** on plasmid DNA electrophoretic mobility was studied in agarose gels. Compound **7** was used as a control. Compounds **1**–**4** all exhibited the characteristic U-shape when treated in this way. At low values of R (the ratio of ligand concentration to DNA base pairs), increasing amounts of ligand results in a decrease in the electrophoretic mobility of the DNA. At a critical R value (R_c) the maximum decrease is observed, and at values of R above R_c electrophoretic mobility increases again. At R values below R_c intercalation into DNA produces an unwinding of the supercoil of plasmid DNA, reducing the number of negative superhelical turns present. Such unwinding reduces the electrophoretic mobility of the plasmid as it becomes less rigid and rod-like. Above R_c, the DNA becomes positively supercoiled. The molecule becomes more compact and electrophoretic mobility increases.

Compounds **1**–**4** all showed a maximum decrease in electrophoretic mobility of about 1·2 mm when the plasmid was allowed to run about 40 mm through the gel. With these small effects it is difficult to distinguish accurately between the different drugs **1**–**4**, but the general trend **3** > **1**, **2** > **4** was observed and this is consistent with the T_m data which suggest that **3** is a better intercalator than **1** and **2** and that **4** is rather less good.

Molecular modelling is difficult to use as a predictive tool in studies of DNA intercalation because energy minimization tends to result in unrealistic distortion of the DNA structure. Both **2** and **3** can be shown to fit readily between DNA base pairs in a double helix, but with **3** there is less steric crowding which could explain its slightly greater affinity for DNA. None of these compounds is completely planar, owing to the $-CH_2-CH_2-$ bridge, which may explain their low affinity as a group.

Some clear points emerge from the antimalarial potency data in Table 2. None of the synthetic compounds is more potent than berberine itself, although compounds **2** and **4** come close. Both **2** and **4** lack bulky substituents which could inhibit DNA binding, which might indicate that DNA binding is a factor in their antimalarial activity. Compound **3**, however, which binds more strongly to DNA than **1**, **2** and **4**, is much less potent as an antimalarial compound. Affinity for DNA is thus not the sole determinant of high potency in this series.

Compound **5** is unique in the series in having four strongly hydrogen-bonding OH groups, rendering the molecule very hydrophilic. Since the intercalative binding site is largely hydrophobic, simple thermodynamic considerations would dictate a lack of affinity for DNA, at least in the centre of the double helix. Antimalarial potency in the range

0·4–0·64 μM for compound **5** confirms that there must be sites of action other than DNA.

This observation is reinforced by a consideration of the antimalarial potencies of compounds **6**–**12**, which have IC50 values in the range 0·35–87·9 μM. None of these analogues shows any affinity for DNA, consistent with an inability to tolerate bulky groups at the intercalation site. However, there must be a site of action which is more tolerant of bulk at position 13, given the IC50 value 0·60 μM for compound **11** which has a fragmental mass at C13 of 149.

We conclude that berberine is an antimalarial alkaloid with a range of biological activities. Until now none of these activities has been correlated with its antimalarial action. These results show, for a range of berberine analogues, a weak correlation between antimalarial activity and intercalation into DNA. None of the analogues shows a strong affinity for calf thymus DNA and several with no demonstrable affinity at all show antimalarial effects at sub-microgram concentrations. However, three of the analogues with some DNA affinity also show the highest antimalarial activity. It is possible that there is a dual mode of action in which DNA binding contributes to one mechanism only.

A feature of the antimalarial data is the correlation between the IC50 ratio for the protoberberines, comparing chloroquine-resistant and -sensitive strains of *P. falciparum*, with the data for chloroquine itself. There appears to be some level of cross-resistance, even though the protozoa have not been previously exposed to the protoberberines.

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References

Bruce-Chwatt, L. J. (1993) Essential Malariology. 3rd edn, Edward Arnold, London, pp 187–188

Cox, F. E. G. (1991) Malaria control. Challenges for chemotherapy and vaccination. Chem. Ind. 15: 533–536

Creasey, W. A. (1979) Biochemical effects of berberine. Biochem. Pharmacol. 28: 1081–1084

d'Alessandro, U., Leach, A., Drakely, C. J., Bennett, S., Olaleye, B. O., Fegan, G. W., Jawara, M., Langerock, P., George, M. O., Targett, G. A. T., Greenwood, B. M. (1995) Efficacy trial of malaria vaccine Spf66 in Gambian infants. Lancet 346: 462–467

Desjardins, R. E., Canfield, C. J., Haynes, J. D., Chulay, J. D. (1979) Quantitative assessment of antimalarial activity in vitro by a semi-automated microdilution technique. Antimicrob. Agents Chemother. 16: 710–718

Ghosh, A. K., Bhattacharyya, F. K., Ghosh, D. K. (1985) *Leishmania donovani*: amastigote inhibition and mode of action of berberine. Exp. Parasitol. 60: 404–413

Kapp, E., Whiteley, C. (1991) Berberine derivatives as anti-leishmanial drugs. J. Enzym. Inhib. 4: 233–243

Kondo, Y. (1976) Organic and biological aspects of berberine alkaloids. Heterocycles 4: 197–219

McCall, D. L. C., Alexander, J., Barber, J., Jaouhari, R. O., Satoskar, A., Waigh, R. D. (1994) The first protoberberine alkaloid analogue with in vivo antimalarial activity. Biorg. Med. Chem. Lett. 4: 1663–1666

O'Neill, M. J., Bray, D. H., Boardman, P., Phillipson, J. D., Warhurst, D. C. (1985) Plants as sources of antimalarial drugs. Part 1. In vitro test method for the evaluation of crude extracts from plants. Planta Med. 51: 394–397

Sixsmith, D. G., Watkins, W. M., Chulay, J. D., Spencer, H. C. (1984) In vitro antimalarial activity of tetrahydrofolate dehydrogenase inhibitors. Am. J. Trop. Med. Hyg. 33: 772–776

Vennerstrom, J. L., Klayman, D. L. (1988) Protoberberine alkaloids as antimalarials. J. Med. Chem. 31: 1084–1087

Vennerstrom, J. L., Lovelace, J. K., Waits, V. B., Hanson, W. L., Klayman, D. L. (1990) Berberine derivatives as antileishmanial drugs. Antimicrob. Agents Chemother. 34: 918–921

Watkins, W. M., Sixsmith, D. G., Chulay, J. D. (1984) The activity of proguanil and its metabolites cycloguanil and *p*-chlorophenylbiguanide against *Plasmodium falciparum* in vitro. Ann. Trop. Med. Parasitol. 78: 273–278

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JOURNAL OF CHROMATOGRAPHY A

Short communication

Separation of pd$(GC)_{12}$ from pd$(AT)_{12}$ by free solution capillary electrophoresis

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Abstract

The two synthetic self complementary oligonucleotides pd$(AT)_{12}$ and pd$(GC)_{12}$ were separated by free solution capillary electrophoresis (CZE) using simple borate buffers. The effects of pH (7.5–9) and the concentration of the buffer (0.03–0.35 *M*) were investigated. Higher pH values and buffer concentrations led to better resolution and longer migration times, the pH having a more pronounced effect on the separation than the concentration of the buffer. It is proposed that the conformation and effective length of the oligonucleotides may have a role in their separation in free solution capillary electrophoresis.

Keywords: Oligonucleotides

1. Introduction

Synthetic oligonucleotides are used extensively in biochemistry and molecular biology as probes for gene isolation, primers for DNA sequencing and for template amplification. The emergence of the antisense nucleotides as potential therapeutic agents [1] has stimulated further research into the synthesis and modification of oligonucleotides [2,3]. Thus fast and reliable methods for the quality control of oligonucleotides and modified analogues are necessary. HPLC (for short oligonucleotides) [4,5] and capillary gel electrophoresis [6–9] have been used.

With the use of a sieving medium, e.g., cross-linked polyacrylamide [7,10], single base resolution (separation of oligonucleotides differing by one nucleotide in length) is readily achievable for oligomers in the range 30–60 nucleotides. If capillary zone electrophoresis (CZE) is used without a sieving medium (free solution) the primary separation mechanism is primarily a function of the charge to mass ratio of the analytes. Since oligonucleotides above ca. 15 bases have almost the same charge to mass ratio, they are expected to have similar electrophoretic mobilities in free solution (CZE) almost irrespective of length [7,11,12] and the presence of a sieving medium would be expected to be necessary to achieve separation. Even more challenging is the separation of oligonucleotides with identical lengths but with different base composition (base specific recognition). With capillary affinity gel electrophoresis (cAGE), in which an affinity ligand is incorporated into the gel matrix, e.g., poly(9-vinyladenine) [13,14], oligomers of different base composition have been separated by their differential interactions.

In this Short communication we describe the separation of two 24-mer oligonucleotides [pd$(AT)_{12}$ and pd$(GC)_{12}$] in free solution CZE with an un-

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was washed after each run for 1 min with distilled water followed by 1 *M* NaOH for 1 min. Oligonucleotides were detected and identified using high speed scanning in the UV region (200–350 nm) and the electropherograms were recorded at 260 nm.

3. Results and discussion

The 24-mer oligodeoxynucleotides $pd(AT)_{12}$ and $pd(GC)_{12}$ were chosen as model intermediate-length self-complementary oligonucleotides that are long enough to complete one hairpin turn to form the B-form DNA helix. Starting with a total borate concentration (TBC) of 0.22 *M* at pH 7.5, little separation was obtained. The order of migration of the oligonucleotides was determined from the UV spectra of the peaks in the region 200–350 nm. As the two oligonucleotides have distinctive UV spectra it was confirmed that $pd(AT)_{12}$ passes the detector before $pd(GC)_{12}$. When the TBC was increased from 0.22 to 0.35 *M* with the pH constant at 7.5 a significant improvement in resolution was obtained with only a small increase in migration time (Table 1). However complete separation of the two oligonucleotides was not achieved even at a TBC of 0.35 *M*.

As sodium tetraborate solutions (0.02 *M*) were adjusted to pH 7.5, 8.0, 8.5 and 9.0 with 0.5 *M* boric acid the resulting TBC changed to 0.22, 0.15, 0.09 and 0.03 *M*, respectively (Table 1). From the results at pH 7.5, it would be expected that these buffer solutions would show an improvement of resolution at higher ionic strengths if the pH had no effect on the separation. However, the separation improved greatly with increasing pH, in spite of decreasing ionic strengths (Table 1), showing that pH changes had a more significant effect on resolution than the ionic strength of the buffer. The selective effect of pH on the separation of oligonucleotides has previously been reported [15] with crosslinked-polyacrylamide-gel filled capillaries, an optimum separation of three different oligonucleotide decamers being achieved at pH 6 [15].

In order to test if the combined effect of higher ionic strengths and higher pH values could lead to better resolution, 0.08 *M* sodium tetraborate solutions were adjusted to pH 7.5, 8.0, 8.5 and 9.0 with 0.5 *M* boric acid giving solutions of 0.35, 0.29, 0.22 and 0.14 (*M*) TBC. The results (Fig. 1) show that the effects of ionic strength and pH can be combined to produce yet better resolution with baseline separation being obtained at pH 8.0, 8.5 and 9.0. Migration times were found to change considerably with changes in pH (Fig. 1). It should be noted that at pH 9 the current was higher than the maximum limit (100 μA) for the applied voltage (25 kV) which resulted in an automatic adjustment of the applied voltage to 20 kV thereby causing a doubling of the migration times and significant band spreading. The differential peak broadening observed in Fig. 1D together with the pH dependence of the UV absorbance of the nucleotide bases [16] are responsible for the observed changes in relative peak height.

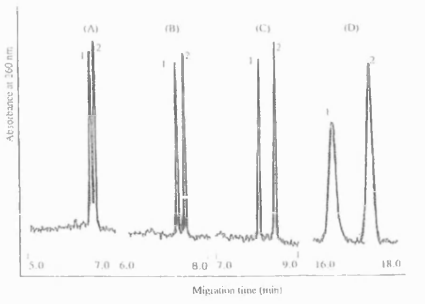

Fig. 1. Sample electropherograms of an equimolar mixture of $pd(GC)_{12}$ and $pd(AT)_{12}$ obtained with 0.08 *M* sodium tetraborate solution adjusted to different pH values (Table 1). (A) pH 7.5, (B) pH 8.0, (C) pH 8.5, (D) pH 9.0. Peaks: 1=$pd(AT)_{12}$, 2=$pd(GC)_{12}$. Conditions: Free solution CZE, applied voltage=25 kV, untreated silica capillary of total length 40 cm and effective length 32 cm.

In all cases increasing the ionic strength of the buffer and/or the pH produced an increase in the average current during the run. Deleterious Joule heating effects on the separation are unlikely except at pH 9 and 0.14 *M* TBC since the capillary temperature was controlled at 20°C and the recorded currents were less than 100 μA.

Baseline separation was obtained without any sieving medium at 0.22 *M* TBC and pH 8.5 in less than 10 min (Fig. 1C). Using several capillaries from different batches, the results were reproducible with only slight differences in resolution from one capil-

lary to another, irrespective of the order of injection (i.e. high to low pH or vice versa). Good migration time reproducibility was obtained at 0.22 *M* borate and pH 8.5 with a relative standard deviation of less than 2.5% for the same capillary.

These results were not expected according to the charge-to-mass ratio concept which is the current basis for explaining separation by CZE, because the two 24-mers would be expected to have the same charge-to-mass ratio in the pH range studied [17]. Moreover, the separation was improved by increasing the ionic strength of the buffer at constant pH (Table 1) which is consistent with the expectation that the separation is not induced by differential ionisation of the oligonucleotides.

It would seem likely that the conformation of the oligonucleotides may have a role in their separation. In a detailed study [18] it has been established that: (a) the minor groove is wide in G:C and mixed sequence B-DNA but narrow in hetero- or homopolymer A:T sequences and (b) propeller twist is low for G:C base pairs and may (but need not) be high for A:T base pairs. More specifically it has been reported that poly d(GC)·poly d(GC) can undergo salt-induced conformational changes [19] which have been shown to be a transition from the A or B right handed conformation to the left handed Z conformation [20]. It has also been found that this transition occurs with poly d(GC)·poly d(GC) but not with poly d(AT)·poly d(AT) [21,22]. Thus differences in the helix conformation and hence overall shape might be responsible for the separation.

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References

[1] G. Zon, Pharm. Res. 5 (1988) 539.
[2] A.S. Cohen, M. Vilenchik, J.L. Dudley, M.W. Gemborys, A.J. Bourque, J. Chromatogr. 638 (1993) 293.
[3] L.A. DeDibiosio, J. Chromatogr. A 652 (1993) 101.
[4] M.J. Gait, Oligonucleotide Synthesis — A Practical Approach, IRL Press, Oxford, 1984, Ch. 5, p. 117.
[5] Y.Z. Xu, P.F. Swann, Anal. Biochem. 203 (1992) 185.
[6] A.S. Cohen, D.R. Najarian, A. Paulus, A. Guttman, J.A. Smith, B.L. Karger, Proc. Natl. Acad. Sci. USA 85 (1988) 9660.
[7] A. Paulus, J.I. Ohms, J. Chromatogr. 507 (1990) 113.
[8] C. Heller, J. Chromatogr. A 698 (1995) 19.
[9] A. Guttman, A.S. Cohen, D.N. Heiger, B.L. Karger, Anal. Chem. 62 (1990) 137.
[10] A. Paulus, E. Gassmann, M.J. Field, Electrophoresis 11 (1990) 702.
[11] A.E. Barron, H.W. Blanch, Sep. Purif. Methods 24 (1995) 1.
[12] G.J.M. Bruin, A. Paulus, Anal. Methods Inst. 2 (1995) 3.
[13] M. Akashi, T. Sawa, Y. Baba, M. Tsuhako, J. High Resolut. Chromatogr. 15 (1992) 625.
[14] Y. Baba, M. Tsuhako, T. Sawa, M. Akashi, Anal. Chem. 64 (1992) 1920.
[15] A. Gutman, A. Arai, K. Magyar, J. Chromatogr. 608 (1992) 175.
[16] G.D. Fasman, Handbook of Biochemistry and Molecular Biology: Nucleic acids, CRC Press, Cleveland, OH, 3rd ed., 1975, p. 419.
[17] G.M. Blackburn, M.J. Gait, Nucleic Acids in Chemistry and Biology, IRL Press, Oxford University Press, 1990, p. 21.
[18] C. Yoon, G.G. Prive, D.S. Goodsell, R.E. Dickerson, Proc. Natl. Acad. Sci. USA 85 (1988) 6332.
[19] F.M. Pohl, T.M. Jovin, J. Mol. Biol. 67 (1972) 375.
[20] S. Arnott, R. Chandrasekaran, D.L. Birdsall, A.G.W. Leslie, R.L. Ratliff, Nature 283 (1980) 743.
[21] A.G.W. Leslie, S. Arnott, R. Chandrasekaran, R.L. Ratliff, J. Mol. Biol. 143 (1980) 49.
[22] A. Mahendrasingham, N.J. Rhodes, D.C. Goodwin, C. Nave, W.J. Pigram, W. Fuller, J. Brahms, J. Vergne, Nature 301 (1983) 335.

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Use of capillary electrophoresis in the study of ligand–DNA interactions

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ABSTRACT

Free solution capillary electrophoresis (FSCE) has been used to separate two non-self-complementary 12mer oligonucleotide duplexes: d(AAATTATATTAT)-d(ATAATATAATTT) and d(GGGCCGCGCCGC)-d(GCGGCGCGGCCC). Titration of mixtures of the two oligonucleotides with model intercalators (ethidium bromide and actinomycin D) and minor groove binders (netropsin, Hoechst 33258 and distamycin) has shown the suitability of FSCE as a method to study the sequence selectivity of DNA binding agents. Binding data have shown cooperativity of binding for netropsin and Hoechst 33258 and have provided ligand:DNA binding ratios for all five compounds. Cooperativity of netropsin binding to a 12mer with two potential sites has been demonstrated for the first time. Ligands binding in the minor groove caused changes in migration time and peak shape which were significantly different from those caused by intercalators.

INTRODUCTION

There is a wide range of established techniques for the study of ligand–DNA binding, from the simple measurement of UV absorption and melting temperature to demanding but highly informative methods using NMR and X-ray crystallography (1–4). In general, separation techniques are not used, with the exception of footprinting, in which gel electrophoresis is used to separate DNA fragments of different lengths and to compare the patterns obtained after DNA cleavage in the presence and absence of a suspected binding species (5–6).

DNA footprinting has recently been improved to allow quantitative comparison of the affinity of a ligand for different base pair sequences. Quantification has demonstrated remarkable selectivity of binding, for example showing a preference for binding of netropsin, distamycin and Hoechst 33258 to AATT and AAAA rather than TTAA and TATA, in contrast to previous general findings of a preference for AT over GC sequences (7). Selectivity of binding is of the utmost importance in designing new ligands for the treatment of disease. Footprinting has been the best method for this purpose for a number of years, but suffers from some experimental disadvantages where large numbers of compounds have to be tested. For simple intercalators the sequence preference is more difficult to detect by standard footprinting methods, since they dissociate rapidly from the binding sites (8–10).

In the present paper we describe a capillary electrophoresis method which is fast, economical and highly informative. The development of such a method for the comparative study of DNA binding affinity, using a competition experiment, required that oligonucleotides of different base pair sequence be separable. While there have been reports of electrophoretic separations based on length, down to 1 bp resolution, using gel-filled capillaries (11), there were no reports of sequence-based separation other than those using affinity labelling (12), which was not suitable for our purpose. In preliminary studies we were surprised and gratified to find that the oligonucleotides $d(AT)_{12}$ and $d(GC)_{12}$ were separable on a simple silica capillary (13), using borate buffer at pH 8.0–8.5.

When we attempted to use $d(AT)_{12}$ and $d(GC)_{12}$ in a model experiment to test whether capillary electrophoresis could be used to detect selective ligand binding, the results were encouraging. Unfortunately, the electropherograms showed multiple peaks for the oligonucleotides in the presence of DNA binding ligands which we attributed to the propensity of these self-complementary oligomers to associate in different ways, i.e. hairpin loops as well as interstrand base pairing. On this basis we designed two directly analogous non-self-complementary AT and GC dodecamers, with a random element in the sequence. The two double-stranded oligomers d(AAATTATATTAT)-d(ATAATATAATTT) and d(GGGCCGCGCCGC)-d(GCGGCGCGGCCC) were used for the ligand binding studies, initially in competition experiments and then singly when it became apparent that a large amount of useful information was obtainable.

MATERIALS AND METHODS

Materials

Boric acid (reagent grade), sodium tetraborate decahydrate (electrophoretic grade), actinomycin D (AcD), ethidium bromide (EtB), distamycin (distamycin A, Dst) and Hoechst 33258 (Ht) were purchased from Sigma Chemical Co. (St Louis, MO). Netropsin (Nt) was a gift from Dr A.R.Pitt (University of Strathclyde). The non-self-complementary oligonucleotides d(AAATTATATTAT)-d(ATAATATAATTT) (AT 12mer) and d(GGGCCGCGCCGC)-d(GCGGCGCGGCCC) (GC 12mer) were obtained as single strands from Cruachem Ltd (Glasgow, UK). Concentrations of the oligonucleotides were measured by

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UV spectrophotometry at 260 nm using the molar absorptivities provided by the supplier. Samples of the oligomers were diluted in distilled water (0.5–1 ml) and stored at –20 C until use. The single-stranded oligonucleotides were mixed in equimolar amounts directly before sample preparation for all experiments.

Capillary electrophoresis (CE) separations

Separations were carried out using a TSP-CE1000 capillary electrophoretic separation system (Thermo Separation Products, USA). Data were acquired and processed using OS/2 Warp v.3 software. Untreated fused silica capillaries (375 µm O.D. and 50 µm I.D.; Composite Metal Ltd, UK) were used with an effective length of 32 cm and a total length of 40 cm. The applied voltage was 25 kV and the capillary temperature was maintained at 20 ± 0.1 C. Oligonucleotides were detected and identified using high speed scanning in the UV region (200–350 nm) and the electropherograms were recorded at 260 nm. Buffer solutions were prepared by adjusting the pH of 0.02 M and 0.08 M sodium tetraborate solutions to 7.5 and 8.0 using 0.5 M boric acid, giving total borate concentrations (TBC) of 0.22 and 0.3 M. Sample and buffer solutions were prepared by milli-Q water purification (Millipore, Bedford, MA) and filtered through 0.2 µm pore size filters (Whatman International Ltd, UK). Free solution capillary electrophoresis (FSCE) showed our sample of AcD to be 87% pure, a figure which was used in subsequent calculations of binding ratios.

Competition experiments

Equimolar mixtures (10 pmol/µl) of each of the AT 12mer and GC 12mer single strands were incubated (1 h) with increasing concentrations of ligand, producing ligand to oligonucleotide (12mer duplex) ratios (r) in the ranges 1:0.0–1:7 for AcD, 1:1–1:15 for EtB and 1:1–1:5 for all minor groove binders (Dst, Nt and Ht). Electropherograms were obtained for all samples with a control sample (zero drug) injected before each run. All injections were made hydrodynamically for 5 s. For EtB, NaCl was added to give a final concentration of 0.02 M in the incubation mixture prior to electrophoresis.

Individual titration experiments

The individual oligonucleotide single strands were incubated for 1 h at room temperature with increasing concentrations of the drug and electropherograms obtained in the same way as for the competition experiments. All experiments were carried out in triplicate (at least) and average values were used to obtain the various plots. Peak height was used rather than peak area because there was not enough resolution to permit peak area quantification; calibration curves were constructed for samples containing increasing concentrations of equimolar mixtures of AT 12mer and GC 12mer to ensure the presence of a quantitative relationship between peak height and the amount of the oligonucleotide and were found to be linear in the range 2.5–20 pmol/µl. The relative standard deviations (RSD) for the peak height method ($n = 10$) of an equimolar mixture of AT 12mer and GC 12mer (at 2.5 pmol/µl) were found to be <5.2% for the AT 12mer and <6.6% for the GC 12mer.

RESULTS AND DISCUSSION

CE is a relatively new analytical technique which is based on the separation of analytes in microcapillaries (10–100 µm I.D.) under the influence of a high electric field. The advantages of CE include speed, quantification, use of an aqueous environment and low sample consumption. Several reports have described the use of CE as a method to study ligand–macromolecule interactions, including drug–DNA (14), drug–protein (15) and antigen–antibody interactions (16). There have been no published studies on the interaction of small molecules with DNA by FSCE.

In order to evaluate the suitability of the FSCE method, well-studied compounds representing the two main types of reversible interaction with DNA (intercalation and minor groove binding) were chosen. AcD, with definite specificity to sequences containing GC base pairs (17,18), and EtB were chosen as typical intercalators. Dst, Ht and Nt, which possess high specificity for AT-containing sequences (7,19), were chosen as minor groove binders.

Two non-self-complementary oligonucleotide duplexes were designed to examine the potential of the technique: d(AAATTA-TATTAT)·d(ATAATATAATTT) (AT 12mer) and d(GGGCCGC-GCCGC)·d(GCGGCGCGGCCC) (GC 12mer). The AT 12mer and GC 12mer were chosen so that: (i) one complete turn of DNA duplex was available for binding; (ii) the non-self-complementary nature of the oligonucleotide duplexes precluded the possibility of hairpin formation, which was found to complicate the analysis (unpublished data); and (iii) the oligonucleotides provided general GC and AT base pair combinations which served as templates for the sequence preference of DNA binding agents. A previously developed method (13) was found to be satisfactory for the separation of the AT 12mer and GC 12mer using borate buffer at pH 7.5 and 0.22 M TBC.

For four of the ligands studied (AcD, Nt, Dst and Ht) it was sufficient to prepare mixtures of the oligomer(s) and ligand in distilled water prior to electrophoresis: in general, peak shape is improved if the sample is of low ionic strength (20). Under these conditions the AT 12mer annealed only when bound to ligand (Nt, Dst or Ht). In the absence of a stabilizing ligand the single AT strands annealed on the column: variation in pH allowed the separation of excess single-strand from duplex, demonstrating duplex formation. UV spectra of eluted AT duplexes complexed with ligands (Fig. 5) were characteristic (21).

AcD did not complex with the AT 12mer, resulting in electropherograms in which the AT was unaffected by increasing concentrations of AcD. Addition of NaCl to the pre-column incubate to bring about AT annealing did not affect this result, the only difference being slight broadening of the oligomer peaks even in the absence of AcD. With EtB it was necessary to add NaCl to the pre-column incubate, otherwise there appeared to be no binding to the AT 12mer, which in distilled water would not anneal even in the presence of EtB.

Intercalators

Actinomycin D. Mixtures and individual samples of the AT 12mer and GC 12mer were titrated with AcD and electropherograms obtained at various drug to oligonucleotide ratios (r); selected electropherograms are shown in Figure 1. As expected (17,18), the electropherograms show selective binding to the GC 12mer with no sign of binding to the AT 12mer. This is inferred from the

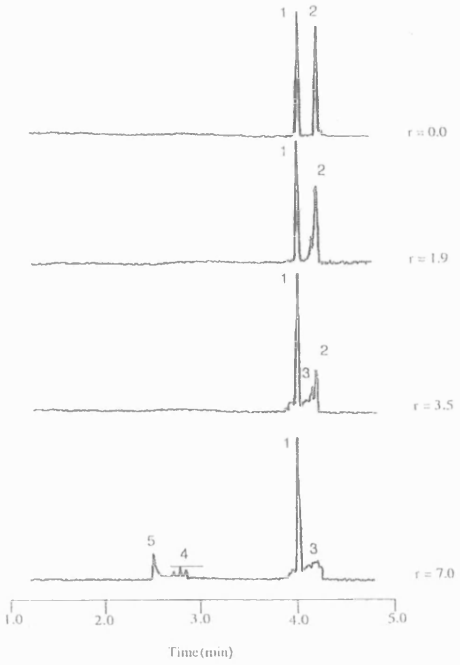

Figure 1. Electropherograms showing the competition experiment of AT 12mer and GC 12mer with actinomycin D. Ratios of AcD per 12mer duplex (r) are indicated. Peaks: 1, free AT 12mer; 2, free GC 12mer; 3, AcD–GC 12mer complex form(s); 4, AcD-related substances; 5, excess AcD. Buffer, 0.22 M TBC, pH 7.5.

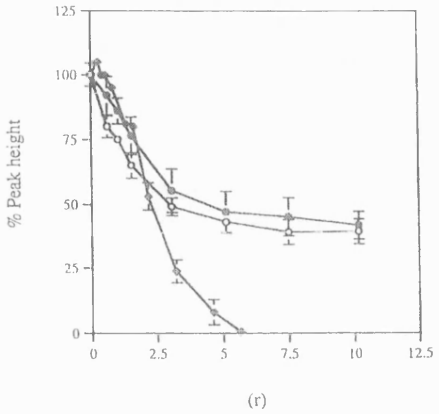

Figure 2. Binding isotherms of actinomycin D (◆) to GC 12mer and ethidium bromide to both AT 12mer (○) and GC 12mer (●) obtained by individual titration experiments. Peak height of the remaining GC 12mer as a percentage of that of a control sample is plotted against r (molar ratio of the drug per GC 12mer). The peak height of GC 12mer sample with no drug (injected immediately before the sample) was taken as 100%. Buffer, 0.22 M TBC, pH 7.5. Error bars show relative standard deviations. For EtB, NaCl (0.02 M) was added to all samples.

gradual disappearance of the GC peak and the emergence of a broad diffuse peak with a shorter migration time than that of the free GC peak. This broad peak was confirmed to be a complex of GC with AcD by UV spectral scanning. The broadness of the peak may be attributable to some dissociation of the complex during passage through the column and/or to different modes of binding.

In order to obtain quantitative binding data titration experiments were carried out for the GC 12mer with AcD. A plot of the percentage peak height of the GC 12mer compared with that of the GC 12mer in a control sample (no drug added) against r provides an estimate of the binding curve (Fig. 2), where r is the molar ratio of the added drug per GC 12mer duplex.

Given that the binding constant of AcD to a representative oligonucleotide was 1.5×10^7/M (22), it is reasonable to assume that all the added drug would be in the bound form up to the saturation point. The stoichiometry of binding can be directly determined from the binding isotherm at the complete disappearance of the free GC peak (Fig. 2), at $\sim r = 5$. However, the broadness of the peak for the complex may indicate different affinities of binding for the five or more molecules of AcD which bind to each GC 12mer, with at least one AcD dissociating during electrophoresis. Possible sites are indicated by parentheses in the sequence : 5′-G()GG()CCG()C-G()CCG()C-3′. Of these, four are expected GC sites and one is an unusual GG site, for which there is a precedent (23). Assuming five binding sites, probability theory predicts that at 40% occupancy there should be 8% free oligomer. Experiment (Fig. 1) shows 57% free oligomer at 38% occupancy ($r = 1.9$), showing a high degree of cooperativity in the binding of AcD to this base pair sequence. The GC 12 mer used in the present study was designed to test the FSCE method: more detailed information about AcD binding could be gained from oligomers with fewer binding sites.

Ethidium bromide. The data for EtB were treated in the same way as for AcD. Earlier studies on EtB reported the compound to have no definite sequence preference (24). However footprinting at 4 C induced marked changes in the pattern of cleavage (10) and the weakest binding was observed for poly(dA) sequences. In the present study, at 20 C, there was no clear preference (Fig. 2).

The binding curve for EtB plateaus above five molecules of EtB to both GC and AT 12mers (Fig. 2). At $r > 5$ no further changes were observed for the oligomer peaks and excess free EtB started to appear in the electropherogram (Fig. 3). There are two notable differences between the electropherograms for AcD and EtB. With AcD, complexes were formed which were sufficiently stable to reach the detector, with different migration times from the free oligomer, so that the height of the free oligomer peak reached zero (Fig. 2) in the presence of sufficient ligand. With EtB, the complex did not survive to reach the detector; dissociation occurred on-column, resulting in peak broadening for both oligomer and ligand. Thus, a clear peak for free EtB only appears above the concentration required to saturate the oligomer (Fig. 3). Beyond this point, there is no further peak broadening effect on the oligomer and the oligomer peak height plateaus at a non-zero value (Fig. 2).

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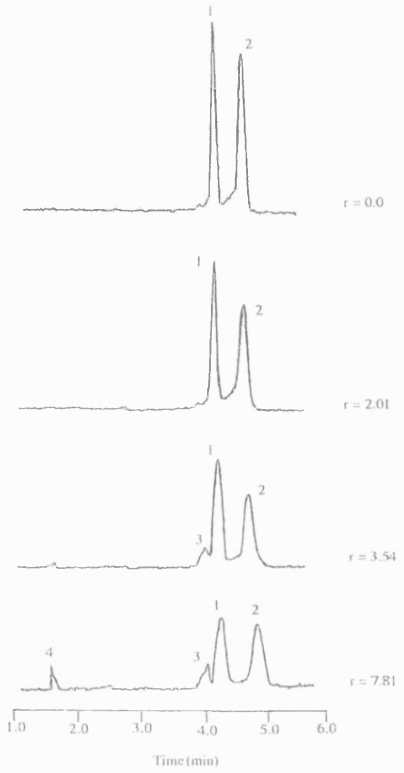

Figure 3. Typical electropherograms for equimolar mixtures of AT 12mer and GC 12mer incubated with increasing concentrations of ethidium bromide. Ratios of EtB per oligonucleotide 12mer duplex (r) are indicated. Peaks: 1, AT 12mer; 2, GC 12mer; 3, unknown; 4, excess EtB. Buffer, 0.22 M TBC, pH 7.5.

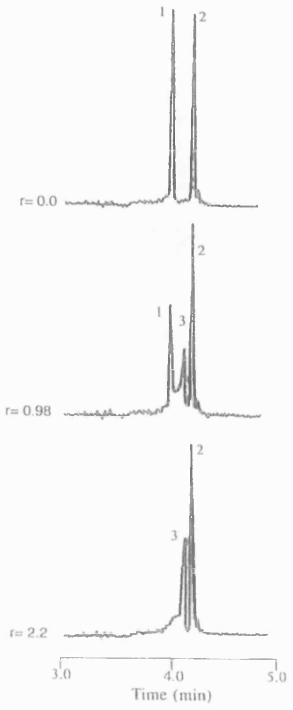

Figure 4. Typical electropherograms for equimolar mixtures of AT 12mer and GC 12mer incubated with increasing concentrations of netropsin. Ratios of netropsin per oligonucleotide 12mer duplex (r) are indicated. Peaks: 1, free AT 12mer; 2, free GC 12mer; 3, netropsin–AT 12mer complex. Buffer, 0.22 M TBC, pH 7.5.

Minor groove binders

The competition experiment for the oligonucleotide mixture with the minor groove binder Nt shows a clear preference for the AT 12mer over the GC 12mer (Fig. 4), as seen from the gradual decrease in the peak area (and height) of the AT 12mer and the appearance of an AT 12mer–drug complex peak until no further AT 12mer remains, while the GC 12mer is not altered. In order to make sure that the GC 12mer did not form complexes which were not separated from the free GC 12mer, UV spectra were obtained at different points on the GC 12mer peak (i.e. peak slicing). These spectra were identical and characteristic of the GC 12mer, indicating the presence of only one species. Since Nt and Dst complexed to DNA are known to exhibit UV maxima at ~320 nm (21), the ability to obtain UV spectra of the eluting peaks is a powerful tool for examining complex formation.

The AT 12mer–Nt complex appears in Figure 4 as a well-defined peak, with a longer migration time than the free AT 12mer, although not separated to baseline. Attempts to achieve complete separation of the complex by changing the concentration of the buffer and/or the pH (7.5–9.0) enhanced the resolution between the free AT 12mer and GC 12mer, but only brought about a slight improvement in the separation of the free and complexed forms of the AT 12mer.

Similar electropherograms to that shown in Figure 4 were obtained for titration of the oligonucleotide mixture with Ht and Dst, showing the clear preference of the drugs for the AT 12mer. In each case the new peak, which had a migration time longer than the free AT 12mer, was confirmed to be the complexed form of the AT 12mer (with either Nt, Dst or Ht) by obtaining the UV spectra (Fig. 5), which showed the characteristics of minor groove binder–DNA complexes (21).

The experiment was repeated with the AT 12mer and GC 12mer separately. In order to obtain quantitative binding data the peak height of the remaining (free) AT 12mer as a percentage of the control AT 12mer (zero drug) was used as a measure of the

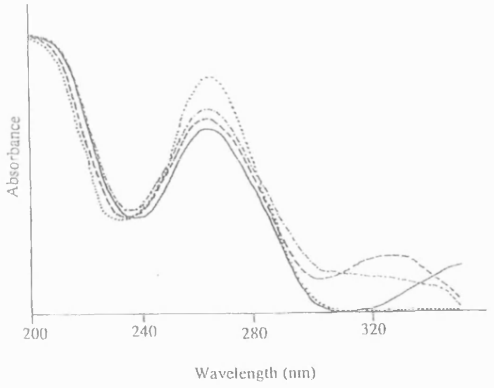

Figure 5. Normalized UV spectra of free AT 12mer (....) and AT 12mer complexed to netropsin (- - -), distamycin (— —) and Hoechst 33258 (—). Buffer, 0.22 M TBC, pH 7.5.

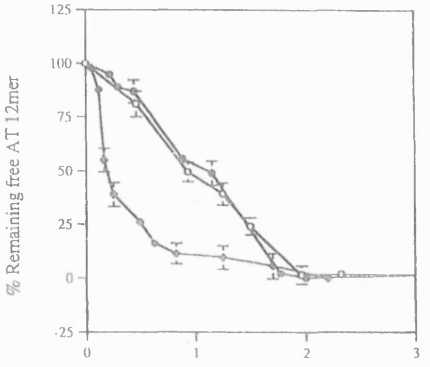

Figure 6. Binding curves of netropsin (○), distamycin (◆) and Hoechst 33258 (●) obtained by titrating AT 12mer with each of the drugs separately. Peak height of a control sample (zero drug) was taken as 100%. Buffer, 0.22 M TBC, pH 7.5. Error bars show relative standard deviations.

remaining free oligonucleotide. A calibration curve was constructed using solutions of equimolar mixtures of AT 12mer and GC 12mer in a similar way to that for EtB and AcD. Binding curves were obtained by plotting the peak height of the AT 12mer in the sample (at each r value) divided by the peak height of the AT 12mer control ($r = 0$), which was injected immediately before the sample, against the molar ratio (r) of the added drug per 12mer (Fig. 6). Since all three minor groove binders (Ht, Nt and Dst) bind strongly to AT-containing oligonucleotides ($K_a = 1 \times 10^{-6}$ to 10^{-9}/M) (25,26), the unbound drug at each of the titration points is negligible, so that the stoichiometry of binding can be obtained directly from the binding curves. The binding curves in Figure 6 show a binding stoichiometry of two drug molecules per AT 12mer for each of the three compounds at saturation, which is in very good agreement with the literature data (27,28). However, the plot in Figure 6 shows different binding isotherms for Nt, Ht and Dst.

Netropsin (Nt) and Hoechst 33258 (Ht). For Nt and Ht the binding isotherms were essentially linear (Fig. 6), which suggests one mode of binding in a cooperative manner. The cooperativity of binding is evident from the binding curve, since at $r = 1$ there was only 50% of the AT 12mer in the bound form. The binding of Nt and Ht to the AT 12mer occurs only in the 2:1 mode, i.e. binding of the first drug molecule facilitates binding of the second. Using NMR techniques it has been demonstrated that Ht binds cooperatively to a 12mer oligonucleotide with two separate binding sites (28). The literature evidence for cooperative binding of Nt is indirect, since there have been no binding studies with similar oligonucleotides containing only two separate binding sites (29).

Distamycin (Dst). For Dst the binding curve was non-linear (Fig. 6), with a saturation binding ratio of 2:1. Unlike Nt and Ht, at $r = 1$ there was >90% of the AT 12mer in the bound form. In this case it is evident that 1:1 binding occurs at lower r values (<1) and 2:1 binding occurs at $r > 1$.

Since Dst requires a binding site of 4–6 bp (27,30,31), the AT 12mer can accommodate two Dst molecules (possibly separated by 1–3 bp). All of the reported studies on Dst–oligonucleotide complexes were performed with oligonucleotides containing single binding sites or with polynucleotides that contain multiple binding sites (29,32).

It is well established that Dst can bind to single binding sites of at least five AT bp in 2:1 (Dst:oligonucleotide) mode (29). In these 2:1 complexes the two Dst molecules bind side-by-side (head-to-tail) in the minor groove of the AT sequence. Molecular mechanics calculations indicate that the minor groove must expand significantly to accommodate two drug molecules side-by-side (33). Since there have been no studies on the binding of Nt and Dst to 12mer oligonucleotides with AT combinations having two potential binding sites, it would be interesting to observe the structural detail of the complex at the molecular level using X-ray or NMR techniques. The present study does not permit a distinction to be made between linear and side-by-side binding.

CONCLUSIONS

CE has been successfully used to probe the sequence preference of DNA binding agents, including minor groove binders and intercalators. In agreement with previous studies, the CE method showed the preference of AcD for a GC-containing oligonucleotide and the preference of the minor groove binders Nt, Dst and Ht for AT-containing sequences, while EtB showed no significant preference.

Since FSCE can separate the free and bound ligands and/or the free and bound oligonucleotides, it is possible to obtain estimates of binding curves and binding ratios. Cooperativity of binding can be directly probed by observing the change in peak height of the free oligonucleotide as a function of drug concentration, and the method showed such binding of Ht to an AT 12mer in agreement with a previous NMR study (28). CE showed cooperative binding of Nt to an AT 12mer and non-cooperative binding of Dst to the same AT 12mer.

An important feature of FSCE is the dependence of the migration time on the conformation of the oligonucleotide or oligonucleotide–drug complex (13). Since intercalating drugs produce changes in DNA secondary structure which are different from those induced by minor groove binders, a systematic difference in the migration time might be expected for intercalators and minor groove binders, provided that the complex survives as a single species during electrophoresis. Such differences in electrophoretic behaviour between minor groove binders and intercalators could provide empirical evidence of the mode of binding of newly designed DNA binding molecules.

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REFERENCES

1 Dougherty,G. and Pigram,W.J. (1982) *CRC Crit. Rev. Biochem.*, **12**, 103–132.
2 Zimmer,C. and Luck,G. (1992) In Hurley,L.H. (ed.), *Advances in DNA Sequence Specific Agents*. JAI Press Inc., London, UK, Vol. 1, pp. 51–88.
3 Searle,M.S. (1993) *Prog. NMR Spectrosc.*, **25**, 403–480.
4 Chaires,J.B. (1992) In Hurley,L.H. (ed.), *Advances in DNA Sequence Specific Agents*. JAI Press Inc., London, UK, Vol. 1, pp. 3–23
5 Galas,D.J. and Schmitz,A. (1978) *Nucleic Acids Res.*, **5**, 3157–3170.
6 Lane,M.J. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 3260–3264.
7 Abu Daya,A., Brown,P.M. and Fox,K.R. (1995) *Nucleic Acids Res.*, **23**, 3385–3392.
8 Fox,K.R. (1992) In Hurley,L.H. (ed.), *Advances in DNA Sequence Specific Agents*. JAI Press Inc., London, UK, Vol. 1, pp. 167–214.
9 Fox,K.R., Waring,M.J., Brown,J.R. and Neidle,S. (1986) *FEBS Lett.*, **202**, 289–294.
10 Fox,K.R. and Waring,M.J. (1987) *Nucleic Acids Res.*, **15**, 491–507.
11 Paulus,A. and Ohms,J.I. (1990) *J. Chromatogr.*, **507**, 113–119.
12 Baba,Y., Tsuhako,M., Sawa,T. and Akashi,M. (1992) *Anal. Chem.*, **64**, 1920–1924.
13 Hamdan,I.I., Skellern,G.G. and Waigh,R.D. (1998) *J. Chromatogr. A.*, **806**, 165–168.
14 Guttman,A. and Cooke,N. (1991) *Anal. Chem.*, **63**, 2038–2042.
15 Kraak,J.C., Bush,S. and Poppe,H. (1992) *J. Chromatogr.*, **608**, 257–264.
16 Heegaard,N.H.H. (1994) *J. Chromatogr. A.*, **680**, 405–412.
17 Scott,E.V., Jones,R.L., Banville,D.L., Zou,G., Miarzilli,L.G and Wilson,W.D. (1988) *Biochemistry*, **27**, 915–923.
18 Goodman,J., Rehfuss,R., Ward,B. and Dabrowiak,J.C. (1992) *Biochemistry*, **31**, 1046–1057.
19 Van Dyke,M.W. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 5470–5474.
20 Jandik,P. and Bonn,C. (1993) *Capillary Electrophoresis of Small Molecules and Ions*. VCH Publishers Inc., New York, NY, pp. 80–82.
21 Zimmer,C., Puschendorf,B., Grunicke,H., Chandra,P. and Wemmer,H. (1971) *J. Mol. Biol.*, **58**, 329–348.
22 Snyder,J.G., Hartman,N.G., D'Estantoit,B.L., Kennard,O., Remeta,D.P. and Breslauer,K.J. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 3968–3972.
23 Waterloh,K. and Fox,K.R. (1992) *Biochim. Biophys. Acta*, **1131**, 300–306, and references cited therein.
24 Krugh,T.R. and Reinhardt,C.G. (1975) *J. Mol. Biol.*, **97**, 133–162.
25 Loontiens,F.G., McLaughlin,L.W., Dickman,S. and Clegg,R.M. (1991) *Biochemistry*, **30**, 182–189.
26 Rentzeperis,D. and Marky,L.A. (1995) *Biochemistry*, **34**, 2937–2945.
27 Luck,G., Triebel,H., Waring,M. and Zimmer,C. (1974) *Nucleic Acids Res.*, **1**, 503–530.
28 Searle,M.S. and Embrey,K.J. (1990) *Nucleic. Acids Res.*, **18**, 3753–3762.
29 Wemmer,D.E., Geierstanger,B.H., Fagan,P.A., Dwyer,T.J., Jacobsen,J.P., Pelton,J.G., Ball,G.E., Leheny,A.R., Bathini,Y., Lown,J.W. *et al.* (1994) In Sarma,R.H. and Sarma,M.H. (eds), *Structural Biology, The State of the Art*. Adenine Press, New York, NY, Vol. 2, pp. 301–323.
30 Klevit,R.E., Wemmer,D.E. and Reid,B.R. (1986) *Biochemistry*, **25**, 3296–3303.
31 Coll,M., Frederick,C.A., Wang,A.H.J and Rich,A. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 8385–8389.
32 Kopka,M.L. and Larsen,T. (1992) In Propst,C.L. and Perun,T.J. (eds), *Nucleic Acid Targeted Drug Design*. Marcel Dekker Inc., New York, NY, pp. 304–371.
33 Pelton,J.G. and Wemmer,D.E. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 5723–5727.

Anti-Cancer Drug Design (1998), **13**, 797–813

The effect of 12-alkoxy modification on the *in vitro* antileukaemic activity of *N*-methyl 2,3,8,9-tetramethoxybenzo[*c*]phenanthridinium salts

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Summary: Some members of a series of 12-alkyloxy benzo[*c*]phenanthridines are potent inhibitors of the growth of P388 tumour cells *in vitro*, with a strong dependence on the nature of the 12-substituent. Analogues with a quaternary nitrogen in the side chain bind strongly to DNA but are less active against the tumour cells. The multi-drug-resistant cell line Pr8/22 shows less sensitivity to the new compounds. K562 Human leukaemia cells undergo differentiation in the presence of the benzo[*c*]phenanthridine derivatives with a structure–activity relationship which does not correlate well with potency against the P388 cell line.

Key words: antileukaemic/benzo[*c*]phenanthridine/DNA binding/synthesis/structure–activity relationship

Introduction

We have described the antileukaemic activity in mice of several benzo[*c*]phenanthridines obtained by a short and efficient chemical synthesis (Olugbade *et al.*, 1990; Olugbade & Waigh, 1996). The previous results showed that antileukaemic potency was heavily dependent on the nature of the 12-substituent, where a hydroxy group abolished activity and methoxy or benzyloxy groups conferred reasonable potency (Olugbade & Waigh, 1996). We now describe the antileukaemic activity of 19 further derivatives, designed according to a model of their probable mode of action.

The natural benzo[*c*]phenanthridines fagaronine and nitidine (Figure 1) have both been shown (Fang *et al.*, 1993; Larsen *et al.*, 1993; Wang *et al.*, 1993) to be inhibitors of topoisomerase I and II; these and other benzo[*c*]phenanthridines have also been shown to

†Deceased. We dedicate this paper to his memory.

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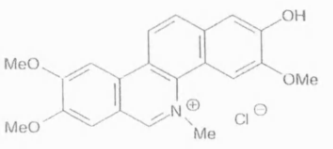
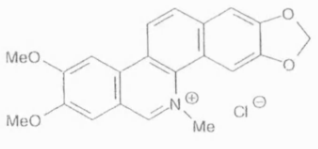
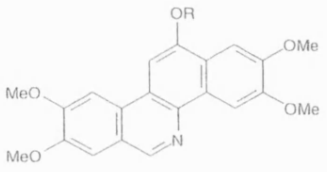
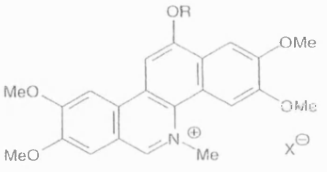


fagaronine 1



nitidine 2



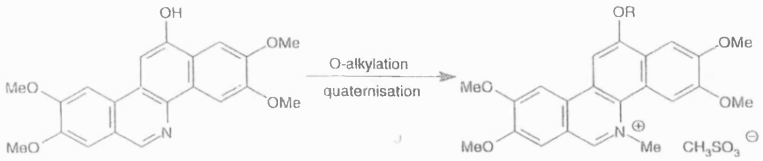


	R =		R =
3	H	21	H
4	CH_3	22	CH_3
5	CH_2CH_3	23	CH_2CH_3
6	$(CH_2)_2CH_3$	24	$(CH_2)_2CH_3$
7	$(CH_2)_3CH_3$	25	$(CH_2)_3CH_3$
8	$(CH_2)_3Br$	26	$(CH_2)_3OSO_2CH_3$
9	$(CH_2)_4Br$	27	CH_2Ph
10	CH_2Ph	28	$(CH_2)_2N^+(CH_3)_3$
11	$(CH_2)_2N(CH_3)_2$	29	$(CH_2)_2N^+(CH_3)Pip$
12	$(CH_2)_2NPip$	30	$CH_2CH(CH_3)CH_2N^+(CH_3)_3$ [a]
13	$CH_2CH(CH_3)CH_2N(CH_3)_2$ [a]	31	$(CH_2)_3N^+(CH_3)_3$
14	$(CH_2)_3N(CH_3)_2$	32	$(CH_2)_4N^+(CH_3)Pip$
15	$(CH_2)_4NPip$	33	$(CH_2)_3Phthal$
16	$(CH_2)_3Phthal$	34	$(CH_2)_4Phthal$
18	$(CH_2)_6$-bis	35	$(CH_2)_3NH_3^+$
19	$(CH_2)_8$-bis	36	$(CH_2)_4NH_3^+$
20	$CH_2COOCH_2CH_3$	37	$(CH_2)_6$-bis
		38	$(CH_2)_8$-bis
		39	$CH_2COOCH_2CH_3$

[a] As racemates

Figure 1 Structures of the tested benzophenanthridines and their precursor bases.

bind to DNA and have been presumed to be intercalators from an assessment of the nature of the polyaromatic nucleus and from UV data (Baez *et al.*, 1983; Pezzuto *et al.*, 1983; Maiti *et al.*, 1984; Smekal *et al.*, 1984; Nandi & Maiti, 1985; Nandi *et al.*, 1985; Kakiuchi *et al.*, 1987). There have been relatively-few studies of synthetic analogues, perhaps because the



3

21 R=H, **22** R=Me, **23** R=Et, **24** R=Pr, **25** R=Bu
26 R=$(CH_2)_3OSO_2CH_3$, **39** R=CH_2COOEt

Figure 2 Products derived from simple *O*-alkylation and quaternization.

available routes were too long (Mackay, 1997); in the present study we have concentrated on the very readily available 2,3,8,9-tetramethoxy series.

Molecular modelling (Mackay, 1990) of the known active 12-benzyloxy derivative showed that the 12-substituent might intrude into the minor groove of DNA, with the tetracyclic nucleus intercalated between the base pairs. The 12-substituent (R in the general structure, Figure 1) was thus an attractive site for molecular modification with a view to optimization of binding of the side chain in the minor groove.

Materials and methods

12-Hydroxy-2,3,8,9-tetramethoxybenzo[*c*]phenanthridine was synthesized as described previously (Olugbade *et al.*, 1990).

Preparation of the 12-(O-alkyl) derivatives of 2,3,8,9-tetramethoxybenzo[c]phenanthridine (Figure 2)

Alkylation with a halide. A mixture of the phenolic benzo[*c*]phenanthridine **3** (600 mg, 1.64 mmol) and 60% sodium hydride in oil dispersion (200 mg, 4.9 mmol) in dry dimethylformamide (10 ml) was stirred under nitrogen for 20 min. The alkyl or benzyl bromide (2.00 mmol) was added and the mixture was stirred under anhydrous conditions for 24 h. The reaction mixture was diluted with 5% sodium hydroxide solution, extracted with chloroform, dried over anhydrous magnesium sulphate, filtered and the solvent removed under vacuum. The residue was redissolved in chloroform (5 ml), added dropwise to acetone and collected by filtration. The properties of the products (**6**, **7** and **10**) are shown in Tables I and II. Thin-layer chromatography of the products on alumina using chloroform:ethanol 9:1 indicated the presence of a single compound in each case.

Alkylation with a dialkyl sulphate. A mixture of the phenolic benzo[*c*]phenanthridine **3** (600 mg, 1.64 mmol) and 60% sodium hydride in oil dispersion (200 mg, 4.9 mmol) was stirred in dry dimethylformamide (10 ml) under nitrogen for 20 min. The dialkyl sulphate (2.0 mmol) was added and the mixture stirred under anhydrous conditions for 24 h. The reaction mixture was diluted with 5% sodium hydroxide solution, cooled and collected by filtration. The

Table I Yields, melting points and MS data for benzo[*c*]phenanthridines

Cpd	*Yield*	*Mp (°C)*	*MS m/z (%) [FAB]*[a]
4	78	270–2	379(100),365(9)
5	85	275	393(100),378(12),364(29)
6	80	261–5	407(33),392(7),378(13),364(33)
7	80	254	421(60),378(18),364(26)
8	73	162–5	487(43),485(42),405(92),364(100)
9	70	186	501(74),499(74),419(18),364(54)
10	62	203–6	455(54),364(100)
11	80	189–193	438(15),437(63),72(100),58(52)
12	83	175	476(3),365(6),112(100),98(43)
13	75	162–5	464(27),463(39),379(14),364(16),100(100)
14	78	180	451(36),450(42),365(28),86(100),58(89)
15	72	178–180	505(6),364(42),140(100),111(16),98(78)
16	87	195	552(55),364(13),188(100),160(84)
17	65	201–4	420(4),160(58)
18	78	167	813(45),365(23)
19	72	171	841(40),365(42)
20	60	176–7	452(93),394(21),364(100)
22	90	235	379(100),364(18) [M,1]
23	92	238	408(4),394(72),364(22) [M,7]
24	90	229	407(66),364(83) [M,3]
25	93	230	421(28),378(13),364(21) [M,7]
26	88	285–290	406(24),364(5) [M,0.1]
27	82	246	455(38),381(19),364(96) [M,0]
28	80	242–6	451(17),406(14),379(9),144(100) [M+$MeSO_3$,5]
29	85	228	476(20),393(13),379(6),365(18),225(26),112(100),98(68) [M,6]
30	88	238	479(15),420(18),379(12),364(25),100(100),58(39) [M,5]
31	90	240	465(20),406(24),364(9),144(100) [M,9]
32	85	255–7	519(9),364(42),140(100),111(21),98(53),84(16) [M–1,3]
33	90	245–6	552(25),365(7),160(47) [M,6]
34	80	251	566(26),365(8),160(84)
35	94	254	436(6),422(17),365(24),58(58),44(100) [M–1,4]
36	92	255–7	452(30),451(100),437(17),408(12),365(16) [M–1,1]
37	70	>310	841(22),827(100),365(52)
38	73	>310	869(24),855(70),840(16),365(55) [M+1,10]
39	80	220	451(90),437(16),365(33),364(30) [M,4]

[a]Measured ion with absolute value of error in ppm.

properties of the products (4 and 5) are listed in Tables I and II. Thin-layer chromatography of the products on alumina using chloroform:ethanol 9:1 indicated the presence of a single compound in each case.

Table II NMR data for 12-substituted benzo[*c*]phenanthridine bases[a]

Cpd	*12-OCH_2-*	*OCH_3*	*Ring CH*	*$N(CH_3)_2$*	*12-alkyl*
4		4.05×3,4.09×2	7.35,7.41,7.72×2,8.72,9.10		
5	4.36,q,7Hz	4.09×2,4.15,4.19	7.35,7.42,7.71×2,8.70,9.11		1.66,t,7Hz (3H)
6	4.33,t,6Hz	4.09×2,4.17,4.19	7.37,7.46,7.74×2,8.71,9.13		1.23,t,8Hz (3H),2.03,m (2H)
7	4,35,t,6Hz	4.09×2,4.16,4.19	7.35,7.43,7.72×2,8.70,9.11		1.10,t,6Hz (3H),1.63–2.04,m (4H)
8	4.55,t,6Hz	4.19,4.18,4.09×2	7.38,7.55,7.66,7.79,8.73,9.15		2.59,m (2H),3.78,t,7Hz (2H)
9	4.39,t,6Hz	4.03×2,4.08×2	7.29,7.55,7.66,7.77,8.70,9.12		1.54–1.97,m (4H),3.42,t,6Hz (2H)
11	4.51,t,8Hz	4.08×2,4.16,4.19	7.36,7.51,7.70,7.74,8.71,9.12	2.51	3.04,t (2H)
12	4.54,t,7Hz	4.08×2,4.17,4.19	7.36,7.49,7.69,7.74,8.71,9.12		1.59,m (6H),2.71,m (4H),3.07,t (2H)
13	4.22,t,6Hz	4.09×2,4.19×2	7.37,7.52,7.74,7.78,8.72,9.13	2.32	1.25,d (3H),1.87,m,2.35,d (2H)
14	4.43,t,6Hz	4.08×2,4.18×2	7.36,7.48,7.71,7.75,8.71,9.12	2.34	2.21,m (2H),2.65,t (2H)
15	4.39,t,6Hz	4.09×2,4.18×2	7.37,7.45,7.71,7.74,8.71,9.12		1.53,m (6H),1.98,m (4H),2.45,m (6H)
16	4.46,t,7Hz	4.09,4.11,4.18,4.19	7.36,7.45,7.59–7.80m (6H),8.78,9.12		2.45,m (2H),4.01,t (2H)
17	4.42,t,6Hz	4.08,4.10,4.19×2	7.36,7.46,7.50–7.78m (6H),8.70,9.15		211,m (4H),3.88,t (2H)
18	4.62,m (4H)	3.97,4.09,4.30,4.40	7.27,7.63,7.79,7.87,8.08,9.33		2.07–2.34,m (8H)
19	4.58,m (4H)	4.15,4.19,4.26,4.38	7.26,7.81,8.06,8.14,9.25		1.77–2.26,m (10H)
20	5.01	4.09,4.11,4.17,4.20	7.35 (2H),7.65,7.79,8.69,9.13		1.34,t,7Hz (3H),4.32,q 7Hz (2H)

[a]Solvent $CDCl_3$ except for **18** and **19**, which were in trifluoracetic acid.

Quaternization of the 12-(O-alkyl) derivatives of 2,3,8,9-tetramethoxybenzo[c]-phenanthridines: general procedure

A mixture of the dry benzo[*c*]phenanthridine base (1.5 mmol), methyl methanesulphonate (3.0 ml) and diisopropylethylamine (0.9 ml) was treated at 170–180°C for 30 min under anhydrous conditions. The reaction mixture was allowed to cool and diluted with dry diethyl ether. The clear supernatant was carefully pipetted off. Absolute ethanol was added to the resinous material and the mixture stirred for 30 min. The yellow suspension was filtered and the remaining yellow powder washed successively with ethanol and dry diethyl ether. Recrystallization was from a mixture of methanol and chloroform (**22**, **27**, **23**, **33** and **34**) or ethanol and chloroform (**24** and **25**). The properties of the products are given in Tables I and III.

Preparation of the 12-(bromoalkoxy)-2,3,8,9-tetramethoxybenzo[c]phenanthridines (Figure 3)

A mixture of the sulphate salt of the phenolic benzo[*c*]phenanthridine **3** (1.5 g, 3.27 mmol) and anhydrous potassium carbonate (1.9 g, 14 mmol) in dry acetone (20 ml) was refluxed for 30 min. The dibromoalkane (17 mmol) was added and the mixture was refluxed for 24 h under anhydrous conditions. The reaction mixture was cooled and the acetone removed under vacuum. The residue was diluted with a 3:1 mixture of water and ethanol (50 ml) and filtered to leave a pale brown product which was washed with 5% sodium hydroxide solution and then with ethanol. The properties of the products (**8** and **9**) are listed in Tables I and II. Thin-layer chromatography of the products on alumina using chloroform:acetonitrile 7:3 indicated the presence of a single compound in each case.

12-(4-Piperidylbutoxy)-2,3,8,9-tetramethoxybenzo[c]phenanthridine 15

A mixture of the 4-bromobutoxy derivative **9** (500 mg, 1 mmol) and piperidine (0.3 ml, 3 mmol) was refluxed in dry acetone (30 ml) for 40 h. The acetone and remaining piperidine were removed under vacuum and the brown powder recrystallized from a mixture of acetone and chloroform to produce a pale buff powder (363 mg, 72%). Thin-layer chromatography of the product on alumina using chloroform: ethanol 9:1 indicated the presence of a single compound, R_f 0.72. Other properties are given in Tables I and II.

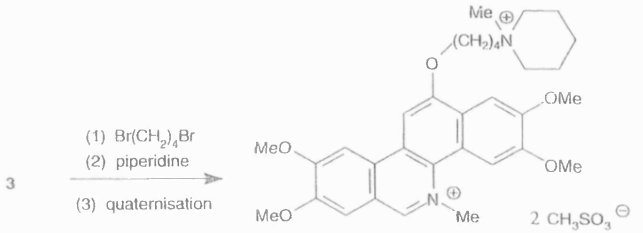
Direct aminoalkylation of the 12-hydroxy-2,3,8,9-tetramethoxybenzo[c]phenanthridine (Figure 4)

A mixture of the sulphate salt of the phenolic benzo[*c*]phenanthridine **3** (500 mg, 1.09 mmol) and anhydrous potassium carbonate (600 mg, 4.5 mmol) in dry acetone (20ml) was refluxed for 30 min under anhydrous conditions. The dialkylaminoalkylchloride hydrochloride (2.0 mmol) was added and the mixture refluxed for 24 h. The acetone was removed under vacuum and the remaining residue diluted with water, stirred for 20 min and filtered to leave a pale brown powder. Recrystallization was from methanol and chloroform (**11**, **13** and **14**) or acetone and chloroform (**12**). The properties of the products formed are listed in Tables I and II. Thin-layer chromatography of the products on alumina using chloroform:ethanol 9:1 indicated the presence of a single compound in each case.

Table III NMR data for 12-substituted benzo[*c*]phenanthridine quaternary salts[a,b]

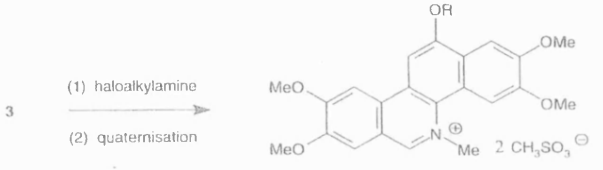
Cpd	*12-OCH_2-*	*OCH_3*	*Ring CH*	*NMe*	*$CH_3SO_3^-$*	*NMe_3*	*12-alkyl*
22		4.24×3,4.38×2	7.75×2,8.14×2,8.19,9.25	5.07	3.17		
23	4.53,q,7Hz	4.19×3,4.31	7.69,7.71,8.07,8.08,8.17,9.18	5.01	3.12		1.72,t,7Hz
24	4.58,t,7Hz	4.31×3,4.43	7.80,7.86,8.18,8.20,8.31,9.30	5.13	3.23		1.37,3H,t,7Hz,2.30,2H,m
25	4.58,t,6Hz	4.24×3,4.36	7.72,8.11,8.14×2,8.23,9.24	5.04	3.17		1.17,t,7Hz,1.73–2.18,4H,m
26	4.68,t,7Hz	4.13×2,4.15,4.28	7.59,7.68,7.82,7.86,7.95,9.15	4.99	3.02[c]		2.28,2H,m,3.10[c],3H,4.15,2H,t
28	5.30,m	4.36,4.40×2,4.54	7.54,8.08,8.15,8.33×2,9.48	5.22	3.38×2	3.69	4.47,2H,m
29	4.85,m	3.87,3.95,4.04,4.15	7.27,7.37,7.48,7.55×2,8.99	4.60	2.72×2		1.69,2H,m,1.96,4H,m,3.20,3H,s,3.50–3.56,4H,m.4.06,2H,m
30	4.63,d,4Hz	4.23,4.26×2,4.38	7.76,7.85,8.03,8.14,8.17,9.30	5.08	3.14×2	3.44	1.70,3H,d,7Hz,2.90,m. 3.74,2H,d,7Hz
31	4.74,t,6Hz	4.26×3,4.38	7.76,8.08,8.14,8.15,8.17,9.29	5.07	3.17×2	3.41	2.94,2H,m,3.98,2H,m
32	4.66,t,6Hz	4.25×3,4.38	7.75,8.08,8.14,8.33×2,9.26	5.07	3.15×2		1.97,6H,m,2.08,4H,m,3.23,3H,s,3.52,6H,m
33	4.51,t,7Hz	4.18,4.24,4.25,4.36	7.73×2,7.84,8.10×2,9.24	5.06	2.98		2.45,2H,4.08,t,6Hz
34	4.52,t,6Hz	4.13×2,4.14,4.28	7.70×2,7.98,8.21×2,9.20	4.95	3.03		2.14,4H,m,3.92,t,6Hz
35	4.68,m	4.25×3,4.37	7.72,8.14,9.20×2,8.16×2	5.08			2.68,2H,m,3.79,2H,m
36	4.62,m	4.26×3,4.39	7.73,8.15,9.24×2,8.16×2	5.07			2.32,4H,m,3.74,2H,m
37	4.67,t,x2	4.02×2,4.25×2,4.28×2,4.39×2	7.81×2,8.18×2,8.19×4,9.30×2	5.13×2	3.21×2		2.08–2.39,8H,m
38	4.50,t,x2	4.10×2,4.18×2,4.20×2,4.31×2	7.69×2,8.06×2,8.09×4,9.18×2	5.03×2	3.09×2		1.14–2.15,10H,m
39	5.50	3.78,4.10,4.13,4.45	6.58,6.76,7.75,7.89,7.99,9.90	4.79	2.57		1.39,3H,t,7Hz,4.38,2H,q,7Hz

[a]All in trifluoracetic acid except **26** in $CDCl_3$/TFA, **29** in D_2O and **39** in $CDCl_3$.
[b]All as methanesulphonates except **35** and **36**, which were chlorides.
[c]Interchangeable.



32

Figure 3 Synthesis of the 4-piperidinobutyloxy analogue **32**.



28 R=$(CH_2)_3N^+(Me)_3$
29 R=$(CH_2)_2N^+(Me)Pip$
30 R=$CH_2CH(Me)CH_2N^+(Me)_3$
31 R=$(CH_2)_3N^+(Me)_3$

Figure 4 Synthesis of derivatives with quaternary ammonium side chains via a haloalkylamine reaction.

Preparation of the 12-(phthalimidoalkoxy)-2,3,8,9 tetramethoxybenzo[c]phenanthridines (Figure 5)

A mixture of the phenolic benzo[*c*]phenanthridine **3** (500 mg, 1.26 mmol) and 60% sodium hydride in oil dispersion (150 mg, 3.67 mmol) in dry dimethylformamide (10 ml) was stirred under nitrogen for 20 min. The *N*-(bromoalkyl)phthalimide (5 mmol) was dissolved in a minimum amount of dry dimethylformamide and added to the mixture, which was then stirred at 80°C for 16 h under anhydrous conditions. The reaction temperature was then reduced to room temperature and stirred for a further 24 h. The mixture was extracted with chloroform, washed with brine, dried over magnesium sulphate, filtered and concentrated *in vacuo* to leave a brown residue. The residue was dissolved in hot acetone followed by precipitation with diethyl ether. The product was collected by filtration. Properties of the reaction products (**16** and **17**) are given in Tables I and II. Thin-layer chromatography of the products on alumina using ethyl acetate:petroleum ether (bp 60–80°C) 9.5:0.5 indicated the presence of a single compound in each case.

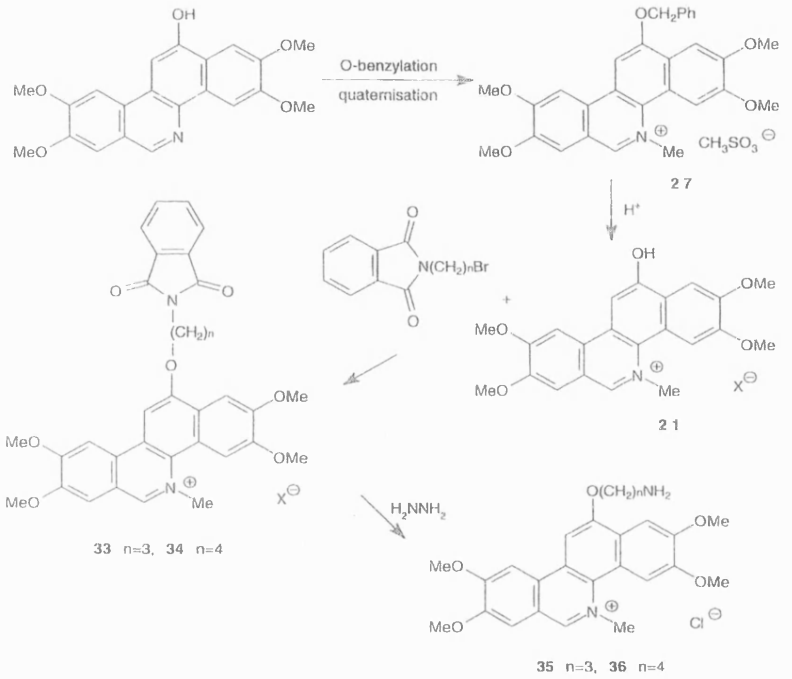


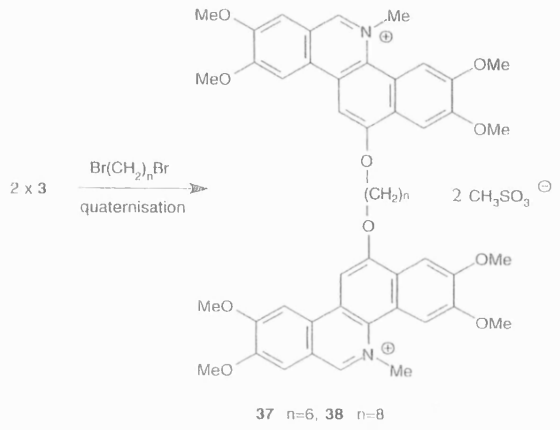
Figure 5 Synthesis of derivatives with side chain amino groups via phthalimide intermediates.

Hydrazinolysis of the phthalimide group

To a boiling solution of the quaternary 12-(phthalimidoalkoxy) derivative (0.38 mmol) in 96% ethanol (10 ml) was added hydrazine hydrate (0.5 ml). On addition of the hydrazine, the yellow suspension immediately dissolved. A precipitate started to form after 3 h of refluxing. After 5 h half of the ethanol was removed under vacuum and the mixture cooled to 0°C. Four drops of conc. HCl and four drops of water were added and the mixture was refluxed for a further 45 min. The solvent was removed under vacuum to leave a yellow powder which was recrystallized from a mixture of ethanol and water. The properties of the products (**35** and **36**) are listed in Tables I and II.

*12-(Ethoxycarbonylmethoxy)-2,3,8,9-tetramethoxybenzo[c]phenanthridine (**20**) and the quaternary salt **39***

The phenolic benzo[*c*]phenanthridine **3** (500 mg, 1.26 mmol) and 60% sodium hydride in oil



37 n=6, **38** n=8

Figure 6 Synthesis of bis-benzophenanthridinium salts.

dispersion (150 mg, 3.67 mmol) in dry dimethylformamide (10 ml) were stirred under nitrogen for 20 min. Ethyl bromoacetate (340 mg, 2.05 mmol) was added and the mixture stirred under anhydrous conditions for 24 h. A further amount of ethyl bromoacetate (340 mg, 2.05 mmol) was added and the mixture stirred for another 24 h. Dilution with water followed by filtration afforded a yellow product (341 mg, 60%), mp 176–177°C. Thin-layer chromatography of the product on alumina using ethyl acetate:chloroform 7:3 indicated the presence of a single compound, R_f 0.60 (the R_f value of the starting material was 0.26). The derivative **20** thus obtained was quaternized according to the general method to yield **39** as a yellow powder (670 mg, 80%), mp 220°C. The properties of **20** and **39** are described in Tables I–III.

Preparation of bis-(2,3,8,9-tetramethoxybenzo[c]phenanthridin-12-yl)dioxaalkanes (Figure 6)

A mixture of dry acetone, anhydrous potassium carbonate (3.0 g, 22 mmol) and the phenolic benzo[*c*]phenanthridine sulphate **3** (2.48 g, 5.4 mmol) was stirred at 75°C for 15 min. The dibromoalkane (2.7 mmol) was added and the mixture gently refluxed for 48 h. The mixture was allowed to cool and the acetone removed under reduced pressure. The residue was diluted with 5% sodium hydroxide and a small amount of acetone, and filtered. After drying, the powder was dissolved in hot chloroform, cooled and precipitated with 96% ethanol. The precipitate was collected by filtration to leave a pale grey powder. The properties of the products (**18** and **19**) are listed in Tables I and II. Thin-layer chromatography of the products on alumina using chloroform:methanol 8:2 indicated the presence of a single compound in each case.

Quaternization of the bis-(2,3,8,9-tetramethoxybenzo[c]phenanthridin-12-yl) dioxaalkanes: general procedure

A mixture of the dry benzo[*c*]phenanthridine base (1.5 mmol), methyl methanesulphonate (6.0 ml) and diisopropylethylamine (1.8ml) was heated at 170–180°C for 1 h under anhydrous conditions. The reaction mixture was allowed to cool and diluted with dry diethyl ether. The clear supernatant was carefully pipetted off. Absolute ethanol was added to the resinous material and the mixture stirred for 30 min. The yellow suspension was filtered and the remaining yellow powder washed successively with ethanol and dry diethyl ether, then recrystallized from a mixture of methanol and chloroform. The properties of the products (**37** and **38**) are given in Tables I and III. The bromopropyl derivative **8**, on treatment with methyl methanesulphonate, underwent substitution of bromine with methanesulphonate to give the sulphonate ester **26**.

Fast atomic bombardment mass spectrometry

Accurate masses for final products (quaternary salts) were obtained using the FAB technique on a Jeol JMS-AX505 HA spectrometer with 3-nitrobenzyl alcohol as matrix. All bis-quaternary salts gave a major ion at $M^+CH_3SO_3^-$; in one case this was the ion accurately measured but in all other examples M^+ or $M^+ \pm 1$ was used, depending on the intensity of the peak.

Thermal denaturation measurements

Highly polymerized calf thymus DNA (type 1) was purchased from Sigma Chemical Co. (Poole, UK) and allowed to hydrate in the required buffer over a period of 1–2 days at 4°C. The concentrations of the DNA solutions were determined spectrophotometrically in terms of nucleotide phosphorus calculated from an extinction coefficient of 6600/M·cm at 260 nm. All experiments were conducted in 0.03 M Tris and 0.018 M NaCl buffer, pH 7.4, adjusted with dilute HCl. Thermal transition profiles were determined using a Philips PU 8800 UV/IIIS spectrophotometer fitted with an Accuron SPX 876 Series 2 Temperature Programme Controller. Measurements were taken at 260 nm with drug:DNA ratios of 1:5 (10 μM drug, 50 μM DNA) in a 3 ml quartz cuvette after equilibration for 20 min. The buffer solution was used in the reference cell and all measurements were duplicated.

Activity against P388 cells in vitro

For cell culture two P388 cell lines were used, one parental and the other (P388R8/22) with acquired resistance to daunorubicin, developed from the parental (P388) line by incremental challenge with the drug *in vitro* (McGown & Fox, 1990). The resistant cell line has been shown to contain amplified gP170 DNA sequence compared with the parental line. All cell lines were grown in RPMI medium supplemented with 10% horse serum (Gibco, Paisley, UK). All cell lines were mycoplasma-free and replaced from frozen stock at 3 month intervals. Cell viability was determined immediately before all experiments and in all cases was >95% as assayed by trypan blue exclusion.

Cell-survival studies were carried out following drug treatment for one cell-cycle period on

cells in exponential growth by back extrapolation of growth curves for the 10 days following drug treatment. All survival experiments were carried out in triplicate.

Cell growth and differentiation of K562 cells

K562 cells were cultured in DMEM medium (Gibco, Cergy Pontoise, France) with 10% fetal bovine serum (Seromed, Berlin, Germany) in flasks at 37°C in a humidified 5% CO_2 atmosphere. Stocks were maintained between 3×10^4 and 8×10^5 cells/ml by dilution into fresh medium three times weekly. Cell growth and cell viability were determined by phase-contrast microscopy.

A stock solution of test compound was made up in ethanol. Drug solutions for testing were obtained by proper dilution with K562 growth medium. The final concentration of ethanol in culture medium never exceeded 0.1%.

K562 cells in exponential growth were plated at a concentration of 5×10^4 cells/ml in multiwell dishes (Nunc, Merelbeke, Belgium). Drug solutions were added at day 0. For 3 days, cell concentration and lethality were evaluated daily in triplicate.

Dose–response curves were used to determine the doses that inhibited growth to 20 (IC_{20}), 50 (IC_{50}) or 80% (IC_{80}) of control growth, or the dose that killed 10% (LC_{10}) of the cells, after 3 days of contact with the drug.

The number of differentiating cells was determined by scoring benzidine-positive cells; K562 were stained using a benzidine–H_2O_2 method in which cells giving an unequivocally positive benzidine reaction, seen as an intense blue cytoplasmic staining, are known to synthesize haemoglobin. An average of 300 cells was scored for benzidine positivity.

Results

The test data for the benzo[*c*]phenanthridines against P388 and K562 leukaemia cells are presented in Tables IV and V. Their effects on DNA melting temperature are presented in Table VI.

Discussion

Chemistry

The simple 12-*O*-alkyl derivatives **22**, **23**, **24** and **25** were synthesized very readily from the general precursor **3** by use of an alkyl halide or sulphate in alkaline solution (Figure 2). The problem of *C*-alkylation at the 11-position (Olugbade & Waigh, 1996) was not observed in any of the present syntheses, except during benzylation to give **27** where the reaction had been previously studied. Following *O*-alkylation at position 12, quaternization was achieved with methyl methanesulphonate as before, using diisopropylethylamine as a proton scavenger. Compound **39** was obtained from **20** in an analogous manner.

For those derivatives in which a nitrogen atom was required in the side chain a variety of approaches were employed. Alkylation of **3** with a large excess of 1,4-dibromobutane gave **9**, which was treated with piperidine to give **15**, which with methyl methanesulphonate (Figure 3) gave the bis-quaternary salt **32**. In contrast, the best route to the piperidine derivative **12** was through 2-chloroethylpiperidine (Figure 4), in a direct reaction with **3**,

Table IV Effect of benzo[*c*]phenanthridines on P388 and P388R8/22 tumour cell lines

Compound	*P388* IC_{50} (μM)	*P388R8/22* IC_{50} (μM)	*Potency ratio*[a]
Fagaronine	0.47		
NDF[b]	inactive		
21	inactive		
22	0.13	4.4	33.8
23	0.08	10.0	125.0
24	0.10	5.7	57.0
25	0.11	2.07	18.8
26	0.07	1.68	24.0
28	7.5	18.3	2.4
29	4.0	10.4	2.6
30	1.6	2.8	1.75
31	4.1	6.8	1.66
32	0.69	ND	
35	4.1	8.6	2.1
36	3.2	11.5	3.6
37	0.2	5.04	25.2
38	0.1	2.1	21.0
39	7.0	9.3	1.33

[a](IC_{50} P388R8/22)/(IC_{50} P388).
[b]*N*-Desmethylfagaronine

giving **29** after quaternization. A similar approach using chloroalkylamines gave **11**, **13** and **14**, which on quaternization gave **28**, **30** and **31**. Alkylation of **3** with a large excess of dibromopropane gave **8**, which was itself quaternized for biological testing; as well as quaternization, **8** underwent substitution of bromine with methanesulphonate to give **26**.

The side chains bearing primary amino groups were obtained by alkylation of **3** with bromoalkylphthalimides to give **16** and **17**, which gave respectively **33** and **34** after quaternization (Figure 5). These two quaternary salts were subjected to hydrazinolysis to give **35** and **36**, which were isolated as the chlorides.

The quaternary bis-benzo[*c*]phenanthridines **37** and **38** were obtained from 1,6-dibromohexane and 1,8-dibromooctane respectively by use of a 2:1 ratio of base to alkyl halide (Figure 6), giving first the bis-bases **18** and **19**, which were quaternized using methyl methanesulphonate as for the simple *O*-alkyl derivatives.

Antileukaemic effects

In the series H → methyl → ethyl → propyl → butyl, the greatest potency against the P388 cell line was observed with ethyl (compound **23**, Table IV), suggesting some hydrophobic bonding and a limiting steric constraint. All four alkylated derivatives showed similar DNA

Table V Effects of benzo[*c*]phenanthridines on human K562 leukaemia cells

Compound	IC_{50} (μM)	LC_{50} (μM)	OIC (μM)	$\%B^+$	RYB^+	*TDI*
Fagaronine	3	30	3.5	70	1	8.6
OMF	6	25	8	10	0.13	3.1
Nitidine	0.4	2	2	60	0.86	1
21	10	10	3	5	0.07	-
22	6	–	–	15	0.21	-
23	5	18	4	80	1.33	4.5
25	1.4	–	–	30	0.43	–
26	1	–	–	7	0.1	–
27	15	–	–	15	0.21	–
28	>10	–	10	3	0.043	–
31	>30	–	–	8	0.11	–
32	>10	>10	10	2	0.029	–
35	39	–	–	15	0.21	–
36	40	–	–	20	0.29	–
38	7	10	10	4	0.057	1
39	>10	–	10	8	0.11	–

Abbreviations: OMF, *O*-methylfagaronine; *IC*, inhibitory concentration; *LC*, lethal concentration; *OIC*, optimal inductive concentration; $\%B^+$, percentage of benzidine-positive cells; RYB^+, relative yield of benzidine positive cells ($\%B^+$ analogue/$\%B^+$ fagaronine); TDI, therapeutic differentiation index (LC_{50}/OIC).

binding properties, as evidenced by changes in 'melting' temperature on complexation (ΔT_m, Table VI).

Modelling showed that a 12-substituent 3–4 atoms long was capable of reaching the negatively charged phosphate backbone, so that incorporation of a positively charged terminal moiety such as an ammonium group in the side chain offered the prospect of improved DNA binding. The final step in the synthesis involves quaternization of the benzo[*c*]phenanthridine nucleus, so the least complicated approach to incorporation of a positive charge into the 12-substituent was the introduction of a tertiary aminoalkyl group which would itself undergo quaternization at the final stage (Figures 3 and 4). Five quaternary ammonium salts, **28**–**32**, were obtained in this way and all showed greatly increased ΔT_m values, in the range 12–15°C (Table VI), implying considerably enhanced DNA binding. However, these quaternary salts showed activity against the P388 cell line (Table IV), which was reduced by 1–2 orders of magnitude compared with the simple ethoxy derivative **23**. The most likely explanation for the reduced potency is that these double-charged derivatives do not penetrate cell membranes very well. In contrast, the single-charged analogues such as **23** can exist in equilibrium with an uncharged 'pseudobase' which should penetrate membranes without difficulty (Simanek & Preininger, 1977; Caolo & Stermitz, 1979; Walterova *et al.*, 1981; Olugbade & Waigh, 1996).

Amino-substituted side chains which are not quaternary salts may exist as free bases in a non-polar environment and according to standard pH-partition theory should pass readily through membranes, while possessing a positive charge in aqueous solution at physiological

Table VI Change of melting temperature of calf thymus DNA at a drug:DNA ratio of 1:5

Compound	ΔT_m (°C)
3	0
5	0
14	0
39	0
33	0
34	0
21	2
23	6
24	6
25	6
27	6
37[a]	6
38[a]	6
22	7
26	8
35	8
36	8
28	12
29	12
30	14
31	14
32	15

[a]Drug:DNA ratio 1:10.

pH. We therefore synthesized the primary amines **35** and **36** by an indirect synthesis through the phthalimides **33** and **34** (Figure 5). However, although both **35** and **36** showed reasonably high affinity for DNA (ΔT_m 8°C), this was not reflected in their potency against P388 cells, which was reduced by 1–2 orders of magnitude compared with the simple alkyl analogues (Table IV). Neither of the phthalimides showed any affinity for DNA (Table VI), perhaps because the side chain substituent is too large; these compounds were not tested against the P388 cell line.

It is known that bis-intercalators may show increased affinity for DNA, with some interesting binding properties, notably the ability to 'walk' along the DNA without detaching (Denny *et al.*, 1985). The 12-OH group offered the prospect of simple *O*-alkyl-*O* linkage (Figure 6), to give bis-benzo[*c*]phenanthridine analogues of the most potent derivative **23**. We chose C_6 and C_8 linking chains, giving derivatives **37** and **38**. Both showed satisfactory DNA binding, giving the same ΔT_m as **23** at half the concentration (Table VI): there were solubility problems with both **37** and **38**. Against the P388 cell line, **37** and **38** were slightly less potent than **23** (Table IV).

With a view to further extension of functionality in the 12-substituent, the ethoxycarbonylmethyl derivative **39** was synthesized as an intermediate. When tested for antileukaemic activity **39** showed low potency and also failed to raise the T_m of calf thymus DNA. In contrast, the propyl derivative with a terminal methanesulphonate group (**26**) showed good affinity (ΔT_m 8°C) and was marginally more potent as an antileukaemic than **23**. It is possible that **26** may benefit from covalent bonding in its interactions with DNA.

As well as the effect on topoisomerase I and II, a known property of fagaronine is the promotion of differentiation in human K562 cells, which can be measured by reaction with benzidine (Comoe *et al.*, 1987, 1988; Benoist *et al.*, 1989). The effects of some of the present compounds are summarized in Table V. The most significant measurement is probably the percentage of benzidine-positive cells, rather than the inhibitory concentration. Both nitidine and fagaronine are very effective in inducing differentiation, although nitidine is considerably more toxic. Compound **23** is more effective than either of the natural products in this assay. While other synthetic compounds in the series are more potent growth inhibitors, none approaches **23** in the more subtle property of differentiation: even the very close analogue **22** has much less effect. Although it is possible to induce differentiation of K562 cells by a variety of chemical agents, including such simple compounds as dimethylsulphoxide, compound **23** does so at very low concentrations. Together with the very unusual structural specificity, the high potency indicates a very high level of target selectivity: these observations are not simply the consequence of DNA binding, which is shown by many less potent members of the series.

Comparing the potency of the benzophenanthridines against P388 cells with their effects on K562 cells, there are some marked discrepancies. Comparing IC_{50} values, compound **26** is one of the most potent in both series (0.07 and 1.0 μM respectively). The relative yield of benzidine positive (RYB^+) cells, however, is very low (0.1) compared with fagaronine (Table V). Compound **22** is fairly close to **23** in potency against P388 cells (0.13 and 0.08 μM respectively) but has an RYB^+ value of 0.21 compared with 1.33 for **23**. Compound **35** has low potency against both P388 (4.1 μM) and K562 cells (39 μM) but an RYB^+ value of 0.21, which is far greater than the RYB^+ value for **32**, which has an IC_{50} against P388 of 0.60 μM. Overall, there is evidence that the inhibitory effects on growth may be mediated by a different mechanism from the induction of haemoglobin production.

The reduced potency shown by many members of the series against the multi-drug-resistant strain Pr8/22 is disappointing. However, we are encouraged by the high potency and the high structural specificity against K562 cells to continue work in this series.

Acknowledgements

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References

BAEZ, A., GONZALEZ, F.A., VASQUEZ, D. & WARING, M. (1983). Interaction between a 3-nitrobenzothiazolo-(3,2-*a*)quinolinium antitumour drug and deoxyribonucleic acid. *Biochemical Pharmacology*, **32**, 2089.

BENOIST, H., COMOE, L., JOLY, P., CARPENTIER, Y., DESPLACES, A. & DUFFER, J. (1989). Comparative effects of fagaronine, adriamycin and aclacinomycin on K562 cell sensitivity for natural-killer-mediated

lysis. Lack of agreement between alteration of transferrin receptor and CD15 antigen expressions and induction of resistance to natural killer. *Cancer Immunology and Immunotherapy*, **30**, 289.

CAOLO, M.A. & STERMITZ, F.R. (1979). Benzophenanthridine salt equilibria. *Heterocycles*, **12**, 11.

COMOE, L., JEANNESSON, P., TRENTESAUX, C., DESOIZE, B. & JARDILLIER, J-C. (1987). The antileukaemic alkaloid fagaronine and the human K562 leukaemic cells : effects on growth and induction of erythroid differentiation. *Leukaemia Research*, **11**, 445.

COMOE, L., CARPENTIER, Y., DESOIZE, B. & JARDILLIER, J-C. (1988). Effect of fagaronine on cell cycle progression of human erythroleukaemia K562 cells. *Leukaemia Research*, **12**, 667.

DENNY, W., ATWELL, G., BAGULEY, B. & WAKELIN, L. (1985). Potential antitumour agents 44. Synthesis and antitumour activity of new classes of diacridines—importance of linker chain rigidity for DNA-binding kinetics and biological activity. *Journal of Medicinal Chemistry*, **28**, 1568.

FANG, S.D., WANG, L.-K. & HECHT, S.M. (1993). Inhibitors of DNA topoisomerase I isolated from the roots of *Zanthoxylum nitidum*. *Journal of Organic Chemistry*, **58**, 5025.

KAKIUCHI, N., HATTORI, M., ISHII, H. & NAMBA, T. (1987). Effect of benzo[*c*]phenanthridine alkaloids on reverse transcriptase and their binding property to nucleic acids. *Planta Medica*, **22**, 22.

LARSEN, A.K., GRONDARD, L., COUPRIE, J., DESOIZE, B., COMOE, L., JARDILLIER, J-C. & RIOU, J-F. (1993). The antileukaemic alkaloid fagaronine is an inhibitor of DNA topoisomerases I and II. *Biochemical Pharmacology*, **46**, 1403.

MACKAY, S.P. (1990). *Synthesis, Antileukaemic Activity and DNA Binding of Derivatives of O-Methylfagaronine*. PhD Thesis, University of Manchester.

MACKAY, S.P., METH-COHN, O. & WAIGH, R.D. (1997). Synthesis of quaternary benzo[*c*]phenanthridine alkaloids and their analogues. *Advances in Heterocyclic Chemistry*, **67**, 345.

MAITI, M., NANDI, R. & CHAUDRI, K. (1984). Interaction of sanguinarine with natural and synthetic deoxyribonucleic acids. *Indian Journal of Biochemistry and Biophysics*, **21**, 158.

MCGOWN, A.T. & FOX, B.W. (1990). Differential cytotoxicity of Combretastatins A1 and A4 in two daunorubicin-resistant P388 cell lines. *Cancer Chemotherapy and Pharmacology*, **26**, 79.

NANDI, R. & MAITI, M. (1985). Binding of sanguinarine to deoxyribonucleic acids of differing base composition. *Biochemical Pharmacology*, **34**, 321.

NANDI, R., CHAUDHURI, K. & MAITI, M. (1985). Effects of ionic strength and pH on the binding of sanguinarine to deoxyribonucleic acid. *Photochemistry and Photobiology*, **42**, 497.

OLUGBADE, T.A. & WAIGH, R.D. (1996). Synthetic benzo[*c*]phenanthridines with antileukaemic activity in mice. *Pharmaceutical Sciences*, **2**, 259.

OLUGBADE, T.A., WAIGH, R.D. & MACKAY, S.P. (1990). A very short route to fully aromatic 2,3,8,9- and 2,3,8,9,12-oxygenated benzo[*c*]phenanthridines. *Journal of the Chemical Society, Perkin Transactions*, 1, 2657.

PEZZUTO, J.M., ANTOSIAK, S.K., MESSMER, W.M., SLAYTOR, M.B. & HONIG, G.R. (1983). Interaction of the antileukaemic alkaloid 2-hydroxy-3,8,9-trimethoxy-5-methylbenzo[*c*]phenanthridine (Fagaronine) with nucleic acids. *Chemico-Biological Interactions*, **43**, 323.

SIMANEK, V. & PREININGER, V. (1977). Pseudobase formation from quaternary pyridinium, quinolinium and isoquinolinium cations. *Heterocycles*, **6**, 475.

SMEKAL, E., KUBOVA, N. & KLEINWACHTER, V. (1984). Interaction of the benzo[*c*]phenanthridine alkaloid sanguinarine with DNA. *Journal of Biochemistry and Biophysics*, **10**, 125.

WALTEROVA, D., ULRICHOVA, J., PREININGER, V. & SIMANEK, V. (1981). Inhibition of liver alanine aminotransferase activity by benzophenanthridine alkaloids. *Journal of Medicinal Chemistry*, **24**, 1100.

WANG, L.-K., JOHNSON, R.K. & HECHT, S.M. (1993). Inhibition of topoisomerase I function by nitidine and fagaronine. *Chemical Research and Toxicology*, **6**, 813.

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The Role of the Iminium Bond in the Inhibition of Reverse Transcriptase by Quaternary Benzophenanthridines

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Abstract

The quaternary benzo[*c*]phenanthridines fagaronine, nitidine and *O*-methylfagaronine have been reviewed as potential antitumour and antiviral agents. Their mode of action has not been established, but their ability to bind with DNA by intercalation is believed to be involved.

Of the three synthetic analogues of *O*-methylfagaronine which we have synthesized, methoxidine and ethoxidine are active against HIV-1 reverse transcriptase (IC50 values 2·8 μM and 2·4 μM respectively) whereas hydroxidine is inactive. One of the prerequisites for the enzyme inhibitory activity of this class of molecule is the presence of an iminium group—it is well known that a positive charge on a polyaromatic nucleus facilitates intercalative binding with DNA. Through UV spectrophotometric and modelling studies, we have shown that the iminium bond plays a more fundamental role in enzyme inhibition through its susceptibility to nucleophilic attack—the inactive analogue hydroxidine has a non-electrophilic iminium bond.

Consequently, we have demonstrated that iminium bond electrophilicity is a parameter which needs to be considered in ternary complex formation with reverse transcriptase.

The pharmacological activity of the benzo[*c*]phenanthridines generally applies to conditions characterized by rapid cell growth (Simeon et al 1989). Sanguinarine (1) and chelerythrine (2) (Table 1) are active against fungal organisms (Hejtmankova et al 1984) and have significant antibacterial activity (Lenfield et al 1981); neither has antitumour activity (Caolo & Stermitz 1979). Fagaronine (3), nitidine (4) and *O*-methylfagaronine (5) have been reviewed as potential antitumour and antiviral agents, but do not have the biological activity of sanguinarine or chelerythrine (Messmer et al 1972; Zee-Cheng & Cheng 1975; Arisawa et al 1984; Comoe et al 1987, 1988). To date, the locus of activity of these analogues has not been established, but their ability to bind with DNA in what appears to be an intercalative mode is believed to be involved in their mechanism of action (Maiti et al 1982, 1984; Pezzuto et al 1983; Nandi & Maiti 1985; Casiano Torres & Baez 1986; Sen & Maiti 1994).

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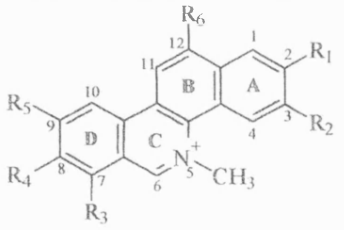
Since the 1970s, the activity of these compounds against a number of DNA-processing enzymes has been assessed (Sethi 1976, 1981, 1984, 1985a, b; Kakiuchi et al 1987; Barret & Sauvaire 1992). A correlation has been established between inhibitory activity against the RNA tumour viruses AMV and MuLV reverse transcriptase (RT) with activity against the P388 leukaemia cell line, because murine leukaemia P388 is an example of the conversion of healthy cells to tumour cells by infection with an oncogenic virus (Poiesz et al 1980). Sethi (1981, 1985a, b) showed that activities were dependent on the different substituent patterns around the benzo[*c*]phenanthridine nucleus, with maximum inhibition of viral DNA activity requiring 2,3,8,9-substituents and an iminium group. Stermitz et al (1975) observed that antitumour activity was confined to this substituent pattern, and that any variation resulted in loss of antitumour activity. RT inhibitory activity was in close agreement with this generalization because

Table 1. The structures of the quaternary benzo[*c*]phenanthridines.



Compound	R_1	R_2	R_3	R_4	R_5	R_6
1	$-OCH_2O-$			$-OCH_2O-$	H	H
2	$-OCH_2O-$		CH_3O	CH_3O	H	H
3	OH	CH_3O	H	CH_3O	CH_3O	H
4	$-OCH_2O-$		H	CH_3O	CH_3O	H
5	CH_3O	CH_3O	H	CH_3O	CH_3O	H
6	CH_3O	CH_3O	CH_3O	OH	H	H
7	CH_3O	CH_3O	H	CH_3O	CH_3O	CH_3O
8	CH_3O	CH_3O	H	CH_3O	CH_3O	CH_2CH_3O
9	CH_3O	CH_3O	H	CH_3O	CH_3O	OH

chelerythrine and sanguinarine were only moderate inhibitors (Sethi 1985). Of significance was the observation that some of the benzo[*c*]phenanthridines formed a ternary complex with the RT and the nucleic acid to which it was bound (Sethi 1984). Fagaronine and nitidine when tested against RT from HIV-1 and HIV-2, showed comparable activity with AMV RT, and were the most potent of 156 pure natural products tested; chelerythrine was inactive (Tan et al 1992).

More recently, topoisomerase enzymes have become a focus for the expression of antitumour activity and it has been shown (Fang et al 1993; Larsen et al 1993) that fagaronine and nitidine stabilized topoisomerase I in a ternary cleavable complex at low concentrations, whereas at higher concentrations they inhibited topoisomerase II, but did not stabilize the cleavable complex.

We have used UV spectrophotometric and molecular modelling studies to propose a mechanism for the inhibition of RT by the benzo[*c*]phenanthridines through the formation of these ternary benzo[*c*]phenanthridine–nucleic acid–enzyme complexes.

Materials and Methods

Chemistry

The quaternary benzo[*c*]phenanthridines hydroxidine, methoxidine and ethoxide were synthesized according to methods developed by Waigh and coworkers (Olugbade et al 1990; Mackay et al 1996, 1998). In summary, the 12-hydroxy-2,3,8,9-tetramethoxybenzo[*c*]phenanthridine (prepared by a four-step synthesis from veratraldehyde and veratrylamine) was treated with the appropriate di alkyl sulphate to produce the corresponding 12-alkoxy ethers, which were subsequently quaternized with methyl methanesulphonate to yield methoxidine and ethoxidine. Hydroxidine was prepared by initially treating the 12-hydroxy-2,3,8,9-tetramethoxybenzo[*c*]phenanthridine with benzyl bromide to yield the 12-benzyloxy analogue; this was quaternized with methyl methanesulphonate and the benzyl ether cleaved by reaction with glacial acetic acid and concentrated hydrochloric acid.

HIV RT Assay

HIV-1 RT (51/66K heterodimer) was expressed in *E. coli* (TG-1) from the recombinant plasmid pRT1 (Larder et al 1987). After a 1 M NaCl extraction procedure the RT was purified by chromatography on an RTMAb8 immunoabsorbent column (Tisdale et al 1988). RT was used to catalyse the incorporation of [^{3}H]thymidine 5′-monophosphate with rAdT (5 μg mL^{-1} or 50 μg mL^{-1}, 3 : 1) used as template/primer. Reactions were incubated for 10 min at 37°C in the presence and absence of the three benzo[*c*]phenanthridines in a range of tenfold dilutions from 100 μM (dissolved in 1% DMSO reaction mixtures consisting of 50 mM Tris-HCl, pH 8·0, 10 mM dithiothreitol, 10 mM $MgCl_2$, 0·05% nonylphenoxy polyethoxy ethanol, 100 μM NaCl and 5 μM TTP, including 0·1 μM [^{3}H]TTP). At the

end of the incubation, reaction mixtures were spotted on to DE81 discs.

The discs were washed to remove unincorporated label and the activity determined by scintillation counting. IC50 values were calculated from the counts.

H9 DNA polymerase α assay

DNA polymerase α, purified from H9 cells (human lymphocytes) as described by Parker et al (1991), was used in assays to catalyse the incorporation of [^{3}H]deoxyAMP; activated calf thymus DNA (200 $\mu g\,mL^{-1}$) was used as template/primer. Reactions were incubated for 30 min at 37°C, in the presence and absence of the three benzo[*c*]phenanthridines, in a range of tenfold dilutions from 100 μM (dissolved in 5% DMSO reaction mixtures consisting of 1 mM dithiothreitol, 5 mM $MgCl_2$, 0·5 $mg\,mL^{-1}$ bovine serum albumin, 60 mM Tris-HCl pH 7·4, 100 μM deoxyCTP, 100 μM deoxyGTP, 100 μM thymidine triphosphate, 200 $\mu g\,mL^{-1}$ activated calf thymus DNA (Pharmacia), and 12 μM [^{3}H]deoxyATP). Reaction mixtures were spotted on to DE81 discs; these were then washed to remove unincorporated label and the activity was determined by scintillation counting. IC50 values were calculated from the counts.

UV spectrophotometric studies

Determination of alkanolamine formation. The benzo[*c*]phenanthridines methoxidine (**7**), ethoxidine (**8**) and hydroxidine (**9**) were prepared as 10–15 mM solutions in doubly-distilled water. pH was measured with a Phillips PW9418 pH meter calibrated beforehand by use of pH buffer reference standards B-4895 (pH 10·0), B-4770 (pH 7·00) and B-5020 (pH 4·01) from Sigma. Absorbance measurements were taken over the range 330 to 450 nm (Uvikon 930 spectrophotometer) at pH 2·0, 8·0 and 12·0 (adjusted by addition of 2 M sodium hydroxide or hydrochloric acid solution to the drug solutions).

DNA binding studies. Concentrations of highly polymerized calf thymus DNA (type 1) from Sigma in 0·03 M Trizma (Sigma) and 0·018 M NaCl buffer, pH 7·4, were determined spectrophotometrically in terms of nucleotide phosphate calculated from an extinction coefficient of 6600 $M^{-1}\,cm^{-1}$ at a wavelength of 260 nm. Hydroxidine, methoxidine and ethoxidine were prepared as stock solutions in buffer and diluted (with buffer) to a final concentration within the range 30–45 μM. Spectrophotometric titrations were performed by adding different volumes of the calf thymus DNA solution to a solution (2 mL) of the drug in a quartz cuvette (3 mL) to vary the benzo[*c*]phenanthridine/DNA phosphate ratio until no further changes in absorbance were measured. Absorbance was measured over the range 330–450 nm (Uvikon 930 spectrophotometer). All measurements were made at 20°C and an equilibration time of 20 min was left after each addition of DNA. The measurements were repeated twice for each benzo[*c*]phenanthridine assayed.

Molecular-modelling studies

Molecular orbital (MO) calculations for the benzo[*c*]phenanthridines were performed using the MOPAC semi-empirical program with graphics visualization using Chem-X (October 1993 version; Chemical Design Ltd, Oxon, UK) and Insight II software (Version 95.0; MSI Technologies, San Diego, CA). Compounds were geometry-optimized using the AM1/MOPAC program, a charge of +1 was assigned to the quaternary nitrogen and −1 to the anionic oxygen of hydroxidine; electrostatic potentials, atom charges, bond-order matrices, $\sigma+\pi$ bonds and π-electron densities were calculated by the AM1/MOPAC semi-empirical method. Electrostatic potential maps of the benzo[*c*]phenanthridines using atom-based point charges determined by AM1 were visualized using the Chem-X graphics interface.

Results

HIV-1 RT inhibition

The enzyme inhibitory assays (Table 2) indicate that two of our synthetic analogues of *O*-methylfagaronine—the 12-alkoxy derivatives methoxidine (**7**) and ethoxidine (**8**)—are potent inhibitors of HIV-1 RT (cf. fagaronine; IC50 21·8 μM; Tan et al 1992). Hydroxidine (**9**) was inactive against both HIV-1 RT and human DNA polymerase α. Both methoxidine and ethoxidine showed some selectivity over human DNA polymerase α.

DNA binding studies

The UV spectra of the 12-alkoxy analogues titrated against DNA show an initial bathochromic and hypochromic change at high drug/DNA ratios, but

Table 2. HIV-1 reverse transcriptase inhibitory activity of the quaternary benzo[*c*]phenanthridines.

Benzo[*c*]phenanthridine	IC50 (μM) against HIV-1 reverse transcriptase	IC50 (μM) against DNA polymerase α
Hydroxidine	Inactive	Inactive
Methoxidine	2·8	31·0
Ethoxidine	2·4	34·0

below ratios of 1·00 (methoxidine) and 0·67 (ethoxidine) a further bathochromic shift occurs with a hyperchromic change, until no further absorption increases were observed below ratios of 0·10 and 0·12, for methoxidine and ethoxidine, respectively. The biphasic nature of the interaction (Figure 1), with no clear isosbestic points, indicates that more than one spectroscopically distinct bound form of the drug is present. These interactions are reminiscent of that of the related alkaloid, coralyne, with DNA (Wilson et al 1976), which is characterized by external association at high coralyne/DNA ratios and intercalation as the ratio decreases below 1·0. The interaction of hydroxidine with DNA also seems to involve a biphasic binding mechanism (Figure 2) with no further changes in absorption observed at a hydroxidine/DNA ratio of 0·11.

Determination of alkanolamine formation

The formation of the alkanolamine form of the benzo[*c*]phenanthridines (Figure 3) at high pH is characterized by collapse of the λ_{max} at 400 nm; this is re-established when the pH is reduced, i.e. there is no absorbance by the alkanolamine form in the long-wave UV band (350–450 nm) (Simanek & Preininger 1977; Caolo & Stermitz 1979; Simanek 1985). These spectral changes are seen for methoxidine (data not shown) and ethoxidine (Figure 4), but not for hydroxidine (Figure 5). For hydroxidine, increasing the pH induces a bathochromic shift of the λ_{max} from 402 to 446 nm, characteristic of phenolic ionization; this again is reversed when the pH is reduced, a spectral change which is not associated with alkanolamine formation. We conclude that the iminium bond in hydroxidine is resistant to nucleophilic attack by the hydroxide ion, with ionization of the phenol group taking precedence over alkanolamine formation in the pH range covered in our study.

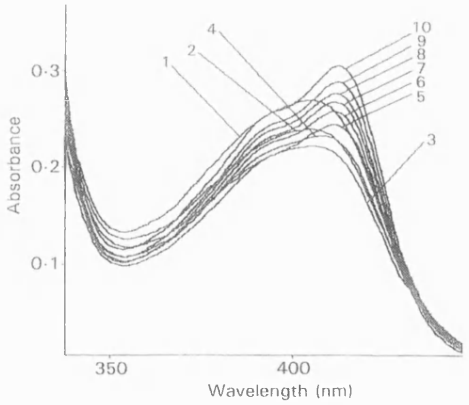


Figure 1. UV absorption spectra of 3·9 μM ethoxidine in 0·018 M NaCl and 0·03 M Tris buffer, pH 7·4, in the absence of DNA (curve 1) and after the addition of calf thymus DNA to yield benzo[*c*]phenanthridine/DNA (phosphate) ratios of 2·86 (curve 2), 1·43 (curve 3), 0·96 (curve 4), 0·58 (curve 5), 0·37 (curve 6), 0·33 (curve 7), 0·3 (curve 8), 0·22 (curve 9) or 0·19 (curve 10). For clarity not all titration curves are shown.

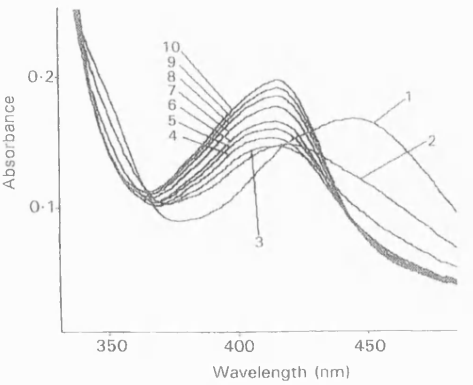


Figure 2. UV absorption spectra of 4·0 μM hydroxidine in 0·018 M NaCl and 0·03 M Tris buffer, pH 7·4, in the absence of DNA (curve 1) and after the addition of calf thymus DNA to yield benzo[*c*]phenanthridine/DNA (phosphate) ratios of 2·94 (curve 2), 1·47 (curve 3), 0·59 (curve 4), 0·43 (curve 5), 0·34 (curve 6), 0·30 (curve 7), 0·22 (curve 8), 0·18 (curve 9) or 0·14 (curve 10). For clarity not all titration curves are shown.

Molecular modelling studies

The electrostatic potential maps of the benzo[*c*]-phenanthridines indicate that the region of highest interaction energy with a unit positive charge is centred around the C-6 position of the iminium bond (>110 kcal mol^{-1}) (Figures 6 and 7) with the exception of hydroxidine. The lowest πz-electron densities for sanguinarine (**1**), chelerythrine (**2**), fagaronine (**3**), *O*-methylfagaronine (**5**) and ethoxidine (**8**) are observed at C-6 (Table 3). The electrostatic potential plot for the phenolate form of hydroxidine (**9**) (Figure 8) is indicative of a molecular electrostatic potential much lower than those of the other benzo[*c*]phenanthridines, tending to be predominantly negative, and a higher πz-electron density at C-6. It seems that the negative charge resident on the oxygen at the 12-position after ionization becomes distributed over the aromatic nucleus increasing the πz-electron density at the 6-position.

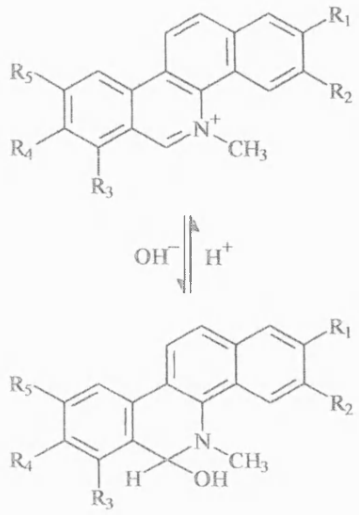


Figure 3. Formation of the alkanolamine form of the benzo[*c*]phenanthridines.

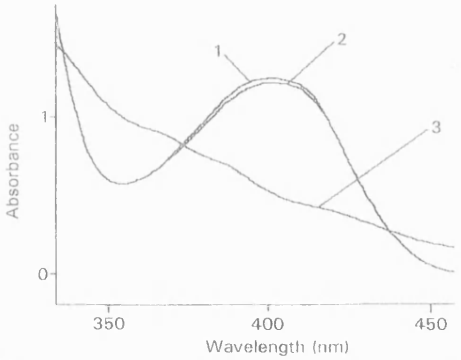


Figure 4. UV absorption spectra of 15·5 mM solutions of ethoxidine at pH 1·4 (curve 1), pH 8·0 (curve 2) and pH 11·4 (curve 3).

Discussion

The quaternary benzo[*c*]phenanthridines occur as two pH-dependent forms (Caolo & Stermitz 1979)—at low pH the cationic species predominates whereas at high pH this species undergoes conversion to the alkanolamine form as a consequence of nucleophilic attack by a hydroxide anion at the electrophilic iminium bond at position 6 of the benzo[*c*]phenanthridine nucleus (Figure 3). The pK_{R+} of this equilibrium, which is analogous to the pK_a value for a Brønsted acid (denoting the pH at which the heterocyclic cation and alkanol-

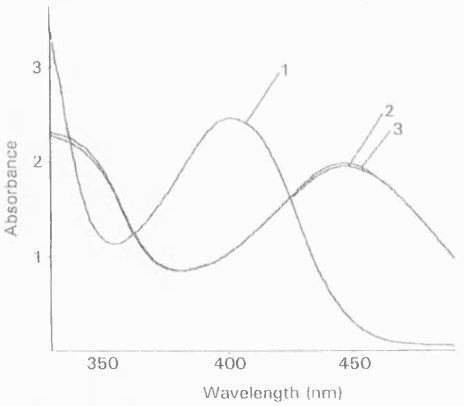


Figure 5. UV absorption spectra of 15 mM solutions of hydroxidine at pH 1·4 (curve 1), pH 8·0 (curve 2) and pH 11·4 (curve 3).

amine are present at equal concentrations) is dependent on the nature and pattern of the oxygenated substituents around the nucleus, with the 7,8-substituted series having lower pK_{R+} values than the 8,9-substituted series (e.g. sanguinarine, 7·32; chelerythrine, 7·53; nitidine, 12·10) (Simanek 1985). The dichotomy in antitumour activity between the active 2,3,8,9-oxygenated and the inactive 2,3,7,8-oxygenated benzo[*c*]phenanthridines has been attributed to these differences in the cation–alkanolamine equilibria. The intercalative binding affinity of sanguinarine for DNA is pH-dependent (Sen & Maiti 1994), this being ascribed to the predominance of the cationic species at low pH (5·2) whereas at higher pH (10·5) the alkanolamine form predominates which, as a consequence of the loss of planarity in the tetracyclic system and the absence of a positive charge on the molecule, does not bind with DNA.

We believe there is a more substantial role for the iminium group in the inhibition of nucleic acid-binding enzymes than the provision of an electrostatic interaction for binding with DNA. We have shown that of the three analogues of *O*-methylfagaronine we have synthesized, methoxidine (**7**), ethoxidine (**8**) and hydroxidine (**9**), two inhibit HIV-1 RT with IC50 values of 2·8 μM (methoxidine) and 2·4 μM (ethoxidine) whereas hydroxidine is inactive. Our DNA binding studies indicate that all three compounds interact with DNA in a biphasic manner reminiscent of the interaction of the related alkaloid coralyne with DNA (Wilson et al 1976). Coralyne is also a potent inhibitor of MuLV and AMV RT (Sethi 1976, 1985a, b) and has a cation–alkanolamine equilibrium (Simanek

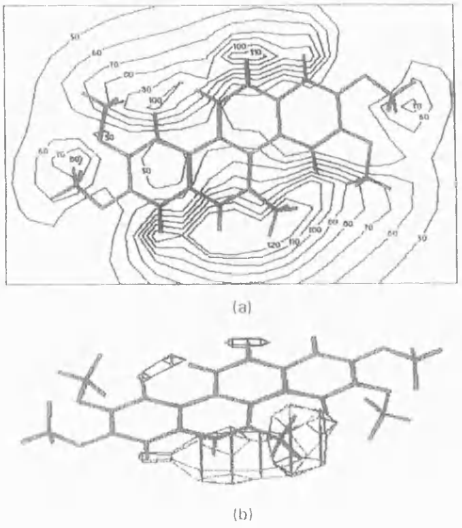


Figure 6. Molecular electrostatic potential map of *O*-methylfagaronine. (a) Electrostatic potential contours through the plane of the molecule with units expressed in kcal mol^{-1}. (b) Electrostatic potential grid showing regions greater than 110 kcal mol^{-1}, predominantly centred over the iminium bond.

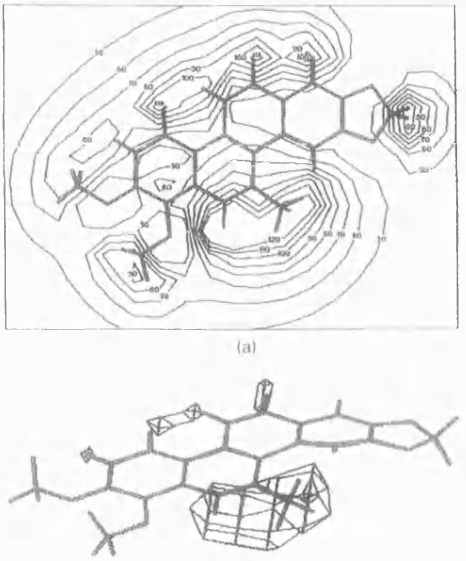


Figure 7. Molecular electrostatic potential map of chelerythrine. (a) Electrostatic potential contours through the plane of the molecule with units expressed in kcal mol^{-1}. (b) Electrostatic potential grid showing regions greater than 110 kcal mol^{-1}, predominantly centred over the iminium bond.

& Preininger 1977). However, of the compounds prepared in this study, only the active 12-alkoxy benzo[*c*]phenanthridines are susceptible to nucleophilic attack at position 6 by the hydroxide anion. We believe that the inhibition of RT and the susceptibility of the iminium bond are linked, and to investigate the electrophilicity of the iminium bond further we have calculated the electrostatic potential maps and the πz-electron densities of the aromatic systems of a series of quaternary benzo[*c*]phenanthridines.

The molecular electrostatic potential is a much better indicator of the electrostatic properties of a molecule than atom-centred charges and can be calculated on a grid surrounding the molecule with contours connecting isopotential points, i.e. points at which the energy of interaction of the molecule with a unit positive charge is identical. Electrostatic potentials can therefore be viewed as a key factor in the control of the long-distance interactions during the approach of a nucleophile to the benzo[*c*]phenanthridine (Grassy et al 1985). Our electrostatic potential maps indicate that an approaching nucleophile would be attracted towards the region of highest interaction energy with a unit positive charge centred around the C-6 position of the iminium bond (>110 kcal mol^{-1}) (Figures 6 and 7) with the exception of hydroxidine. When these

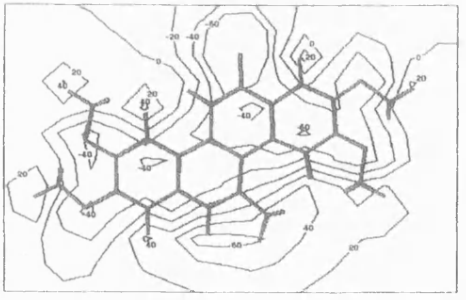
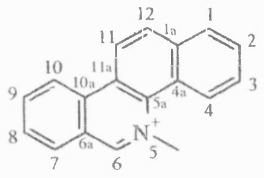


Figure 8. Molecular electrostatic potential contour map of the hydroxidine anion through the plane of the molecule with units expressed in kcal mol^{-1}.

results are viewed in association with the calculated πz-electron densities of the aromatic systems, an indication of the site of electrophilicity within the benzo[*c*]phenanthridine can be deduced (Meunier et al 1988). Because the lowest πz-electron densities for sanguinarine (**1**), chelerythrine (**2**), fagaronine (**3**), *O*-methylfagaronine (**5**) and ethoxidine (**8**) are observed at C-6 (Table 3), we can

Table 3. πz-Electron densities of the quaternary benzo[*c*]phenanthridines.



Atom	Benzo[*c*]phenanthridine π z-electron density of compound					
	1	2	3	5	8	9
1	1·0171	1·0195	1·0311	1·0311	1·0185	0·9291
2	0·9748	0·9764	0·9469	0·9469	0·9744	1·0226
3	0·9437	0·9493	0·9551	0·9551	0·9218	0·9713
4	1·0501	1·0504	1·0671	1·0671	1·0495	1·0575
4a	1·0172	1·0208	1·0367	1·0367	1·0082	1·0031
5a	1·0658	1·0582	1·0376	1·0375	1·0842	1·1660
5	1·4108	1·4237	1·4307	1·4307	1·4129	1·3672
6	0·8219	0·8115	0·8596	0·8596	0·8783	1·0349
6a	1·1305	1·1318	1·0901	1·0901	1·0805	1·0404
7	0·9696	0·9409	1·0222	1·0222	1·0251	1·0265
8	1·0154	1·0239	0·9665	0·9665	0·9632	0·9858
9	0·9306	0·9567	0·9024	0·9024	0·9187	0·9832
10	1·0081	1·0185	1·1077	1·1077	1·0991	0·9882
10a	0·9655	0·9567	0·9235	0·9235	0·9452	0·9773
11a	0·9924	0·9984	1·0081	1·0081	0·9676	0·9729
11	1·0137	1·0111	1·0208	1·0207	1·1487	1·1614
12	0·9302	0·9378	0·9359	0·9359	0·8798	0·7677
12a	0·9921	0·9915	0·9884	0·9884	1·0359	1·0259

*Calculated as the phenolate anion

conclude that nucleophilic attack will be directed towards this site in these compounds. The 2,3,7,8-substituted benzo[*c*]phenanthridines have the lowest πz-electron densities at C-6; this is in accord with their greater susceptibility to attack by the hydroxide anion (Caolo & Stermitz 1979; Simanek 1985; Dostal & Potacek 1990). The electrostatic potential plot for the phenolate form of hydroxidine (**9**) (Figure 8) shows the molecular electrostatic potential to be much lower than those of the other benzo[*c*]phenanthridines; it also shows that the πz-electron density at C-6 is higher, indicating that it has a non-electrophilic iminium bond resistant to nucleophilic attack by the hydroxide ion, as shown by our spectroscopic studies. The electron density at C-12 is low, but in the absence of a corresponding high positive potential ($-60\,kcal\,mol^{-1}$), nucleophilic attack at this position is unlikely. Our conclusion therefore, is that RT inhibitory activity is linked to the presence of an electrophilic iminium bond in the quaternary benzo[*c*]phenanthridines.

Other workers have shown that the iminium bond can be attacked by carbon, nitrogen and sulphur nucleophiles (Dostal & Potacek 1990) and have suggested a role for in-vivo hydration, alkylation or addition at position 6 by certain biological species (Zee-Cheng & Cheng 1975). Studies with AMV and MuLV RT indicate that the inhibitory action of fagaronine and *O*-methylfagaronine is associated with the formation of a labile enzyme–nucleic acid–alkaloid ternary complex (Sethi 1984). The model for inhibition which we propose is that on complexing with the nucleic acid substrate the quaternary benzo[*c*]phenanthridine is bound in such a way that it undergoes nucleophilic attack by a residue within the enzyme (such as cysteine, serine, threonine or tyrosine) when it binds to the substrate-benzo[*c*]phenanthridine complex. The resulting ternary complex is stabilized by a labile covalent bond between the enzyme and the benzo[*c*]phenanthridine which, in turn, is bound to the nucleic acid substrate and enzyme inhibition is effected. Hydroxidine, with a non-electrophilic iminium bond, would be unable to undergo this complexation process and therefore enzyme inhibition would not occur.

Electrostatic potential plots and πz-electron densities for sanguinarine and chelerythrine also revealed electrophilicity at the iminium bond. The weak RT inhibitory activity associated with these alkaloids might be a consequence of the assays being performed at pH 7·0, where a significant percentage of the compounds would exist as the alkanolamine form, thus reducing their affinity for the template-primers (Sen & Maiti 1994).

The absence of inhibitory activity of fagaronine, *O*-methylfagaronine and nitidine with nucleic acid substrates containing only G and C (even though binding is evident) (Sethi 1981, 1984, 1985a, b; Kakiuchi et al 1987) suggests that iminium bond electrophilicity is not the only factor governing enzyme inhibition. Enzyme inhibition might be influenced by the orientation of the benzo[*c*]phenanthridine when bound between the base pairs of the nucleic acid substrate; this, in turn, might be a function of base-pair composition. If inhibition requires the formation of a covalent bond with a residue in the enzyme, then the relative position of the iminium bond will be important in terms of proximity to attack. The macromolecular structure of the enzyme bound with a particular nucleic acid substrate might exert considerable influence on the geometry of the bound benzo[*c*]phenanthridine, and modelling studies are currently in progress to investigate these complexes. We must also consider the electronic effects of different neighbouring base-pairs on the electronic charge distribution of the bound drugs, particularly considering the proposed role for the iminium bond, for which we are also developing appropriate models.

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References

Arisawa, M., Pezzuto, J. M., Bevelle C., Cordell, G. A. (1984) Potential anticancer agents XXXI. *N*-Demethylation of fagaronine. J. Nat. Prod. 47: 453–458

Barret, Y., Sauvaire, Y. (1992) Fagaronine, a novel antileukaemic alkaloid. Phytother. Res. 6: 59–63

Caolo, M. A., Stermitz, F. R. (1979) Benzophenanthridine salt equilibria. Heterocycles. 12: 11–15

Casiano Torres, C. A., Baez, A. (1986) Effects of the antitumor drugs 3-nitrobenzothiazolo[3,2-*a*]quinolinium and fagaronine on nucleic acid and protein synthesis. Biochem. Pharmacol. 35: 679–685

Comoe, L., Jeannesson, P., Trentesaux, C., Desoize B., Jardillier, J.-C. (1987) The antileukemic alkaloid fagaronine and the human K562 leukemia cells: effects on growth and induction of erythroid differentiation. Leuk. Res. 11: 445–451

Comoe, L., Carpentier, Y., Desoize, B., Jardillier, J.-C. (1988) Effect of fagaronine on cell cycle progression of human erythroleukemia K562 cells. Leuk. Res. 12: 667–672

Dostal, J., Potacek, M. (1990) Quaternary benzo[*c*]phenanthridines. Collect. Czech. Chem. Commun. 55: 2841–2873

Fang, S.-D., Wang, L.-K., Hecht, S. M. (1993) Inhibitors of DNA topoisomerase I isolated from the roots of *Zanthoxylum nitidum*. J. Org. Chem. 58: 5025–5027

Grassy, G., Bonnafous, M., Loiseau, P., Adam, Y. (1985) Visualization of molecular electronic effects. Trends Pharmacol. Sci. 6: 57–59

Hejtmankova, N., Walterova, D., Preininger, V., Simanek, V. (1984) Antifungal activity of quaternary benzo[*c*]phenanthridine alkaloids from *Chelidonium majus*. Fitoterapia 55: 291–296

Kakiuchi, N., Hattori, M., Ishii, H., Namba, T. (1987) Effect of benzo[*c*]phenanthridine alkaloids on reverse transcriptase and their binding properties to nucleic acids. Planta Med. 53: 22–27

Larder, B. A., Purifoy, D. J. M., Powell, K. L., Darby, G. (1987) AIDS virus reverse transcriptase defined by high level expression in *Escherichia coli*. EMBO J. 6: 3133–3137

Larsen, A. K., Grondard, L., Couprie, J., Desoise, B., Comoe, L., Jardillier, J.-C., Riou, J.-F. (1993) The antileukaemic alkaloid fagaronine is an inhibitor of topoisomerase I and II. Biochem. Pharmacol. 46: 1403–1412

Lenfield, J., Kroutil, M., Marsalek, E., Slavik, J., Preininger, V., Simanek, V. (1981) Anti-inflammatory activity of quaternary benzophenanthridine alkaloids from *Chelidonium majus*. Planta Med. 43: 161–163

Mackay, S. P., Meth-Cohn, O., Waigh, R. D. (1996) Synthesis of quaternary benzo[*c*]phenanthridine alkaloids and their analogues. Adv. Heterocycl. Chem. 67: 345–387

Mackay, S. P., Comoe, L., Desoise, B., Duval, O., Jardillier, J.-C., Waigh, R. D. (1998) The effect of 12-alkoxy modification on the in vitro antileukaemic activity of *N*-methyl-2,3,8,9-tetramethoxybenzo[*c*]phenanthridinium salts. Anticancer Drug Design 13: 797–813

Maiti, M., Nandi, R., Chaudhuri, K. (1982) Sanguinarine: a monofunctional intercalating alkaloid. FEBS Lett. 142: 280–284

Maiti, M., Nandi, R., Chaudhuri, K. (1984) Interaction of sanguinarine with natural and synthetic deoxyribonucleic acids. Indian J. Biochem. Biophys. 21: 158–165

Messmer, W. M., Tin-Wa, M., Fong, H. H. S., Bevelle, C., Farnsworth, N. R., Abraham, D. J., Trojanek, J. (1972) Fagaronine, a new tumor inhibitor isolated from *Fagara zanthoxyloides* Lam. (Rutaceae). J. Pharm. Sci. 61: 1858–1861

Meunier, G., De Montauzon, D., Bernadou, J., Grassy, G., Bonnafous, M., Cros, S., Meunier, B. (1988) The bioxidation of cytotoxic ellipticine derivatives: a key to structure-activity relationship studies? Mol. Pharmacol. 33: 93–102

Nandi, R., Maiti, M. (1985) Binding of sanguinarine to deoxyribonucleic acids of differing base composition. Biochem. Pharmacol. 34: 321–324

Olugbade, T. A., Waigh, R. D., Mackay, S. P. (1990) A very short route to fully aromatic 2,3,8,9- and 2,3,8,9,12-oxygenated benzo[*c*]phenanthridines. J. Chem. Soc., Perkin Trans. 2657–2660

Parker, W. B., White, E. L., Shaddix, S. C., Ross, L. J., Buckheit, R. W., Germany, J. M., Secrist, J. A., Vince, R., Shannon, W. M. (1991) Mechanism of inhibition of human immunodeficiency virus type-1 reverse transcriptase and human DNA polymerase α, polymerase β

and polymerase γ by the 5′-triphosphates of carbovir, 3′-azido-3′-deoxythymidine, 2′,3′-dideoxyguanosine and 3′-deoxythymidine—a novel RNA template for the evaluation of antiretroviral drugs. J. Biol. Chem. 266: 1754–1762

Pezzuto, J. M., Antosiak, S. K., Messmer, W. M., Slaytor, M. B., Honig, G. R. (1983) Interaction of the antileukemic alkaloid, 2-hydroxy-3,8,9-trimethoxy-5-methylbenzo[*c*] phenanthridine (fagaronine), with nucleic acids. Chem.-Biol. Interact. 43: 323–339

Poiesz, B. J., Ruscetti, F. W., Gazdar, A. F., Bunn, P., Minna, J., Gallo, R. C. (1980) Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. Proc. Natl Acad. Sci USA 77: 7415–7419

Sen, A., Maiti, M. (1994) Interaction of sanguinarine iminium and alkanolamine form with calf thymus DNA. Biochem. Pharmacol. 48: 2097–2102

Sethi, V. S. (1976) Inhibition of mammalian and oncornavirus nucleic acid polymerase activities by alkoxybenzophenanthridine alkaloids. Cancer Res. 36: 2390–2395

Sethi, M. L. (1981) Screening of benzophenanthridine alkaloids for their inhibition of reverse transcriptase activity and preliminary reports on the structure-activity relationships. Can. J. Pharm. Sci. 16: 29–34

Sethi, M. L. (1984) Inhibition of reverse transcriptase activity by benzophenanthridine alkaloids. J. Med. Chem. 27: 544–547

Sethi, M. L. (1985a) Enzyme Inhibition VIII: mode of inhibition of reverse transcriptase activity by analogues, isomers and related alkaloids of coralyne. J. Pharm. Sci. 74: 889–891

Sethi, M. L. (1985b) Comparison of inhibition of reverse transcriptase and antileukemic activities exhibited by protoberberine and benzophenanthridine alkaloids and structure–activity relationships. Phytochemistry 24: 447–454

Simanek, V. (1985) Benzophenanthridine alkaloids. In: Brossi, A. (ed.) The Alkaloids, Vol. 26, Academic Press, New York, pp 185–240

Simanek, V., Preininger, V. (1977) Pseudobase formation from quaternary pyridinium, quinolinium and isoquinolinium cations. Heterocycles 6: 475–497

Simeon, S., Rios, J. L., Villar, A. (1989) Pharmacological activities of benzophenanthridine and phenanthrene alkaloids. Pharmazie 44: 593–597

Stermitz, F. R., Gillespie, J. P., Amoros, L. G., Romero, R., Stermitz, T. A., Larson, K. A., Earl, S., Oyz, J. E. (1975) Synthesis and biological activity of some antitumour benzophenanthridinium salts. J. Med. Chem. 18: 708–713

Tan, G. T., Miller, J. F., Kinghorn, A. D., Hughes, S. H., Pezzuto, J. M. (1992) HIV-1 and HIV-2 reverse transcriptases: a comparative study of sensitivity to inhibition by selected natural products. Biochem. Biophys. Res. Commun. 185: 370–378

Tisdale, M., Ertl, P., Larder, B. A., Purifoy, D. J. M., Darby, G., Powell, K. L. (1988). Characterization of human immunodeficiency virus type-1 reverse transcriptase by using monoclonal antibodies—role of the C-terminus in antibody reactivity and enzyme function. J. Virol. 62: 3662–3667

Wilson, W. G., Gough, A. N., Doyle, J. J. (1976) Coralyne. Intercalation with DNA as a possible mechanism of antileukaemic action. J. Med. Chem. 19: 1261–1263

Zee-Cheng, R. K.-Y., Cheng, C. C. (1975) Preparation and antileukaemic activity of some alkoxybenzo[*c*]phenanthridinium salts and corresponding dihydro derivatives. J. Med. Chem. 18: 66–71

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DNA Binding by Fagaronine and Ethoxidine, Inhibitors of Human DNA Topoisomerases I and II, Probed by SERS and Flow Linear Dichroism Spectroscopy†

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Raman, surface-enhanced Raman scattering (SERS), and flow linear dichroism (FLD) spectroscopies were employed to study the potent anticancer agent fagaronine (FGR, NSC 157995) and its derivative ethoxidine (ETX)—inhibitors of DNA topoisomerases (topos) I and II (Figure 1)—and their complexes with DNA. The FLD data obtained suggest that both compounds are strong major groove intercalators with stoichiometries 1 FGR/2.0 DNA bp and 1 ETX/4.0 DNA bp The SERS spectra of both compounds were recorded at the concentrations down to 10^{-8} M for FGR and 10^{-6} M for ETX, and the SERS-active modes were assigned by comparison of Raman and SERS spectra of the drugs following the changes induced by deuteration and pH environment. The SERS-active surface was proved not to affect the drug/DNA interactions, since the DNA binding constants calculated from the SERS experiments were found to be practically the same as those determined previously by viscosimetric measurements. The SERS study of the FGR/DNA complex showed that the OH group of FGR plays a key role in DNA binding, most probably because of formation of the H bond with DNA. Cooperative use of Raman, SERS, and FLD techniques enabled us to propose a molecular model for drug/DNA interactions. The differences in DNA binding by FGR and ETX are discussed in terms of different topoisomerases inhibitory activities of these drugs.

Introduction

Elucidation of the structure−function relationships and search of the key structural determinants mediating the mechanisms of action of antitumor drugs seem to be one of the most important problems in anticancer research.[1] Selective, sensitive, and nondestructive physicochemical and particularly optical spectroscopic approaches enable us to determine molecular groups of drugs and their targets (DNA, proteins, membranes) and to propose the ways for design of new molecules with desired functions. Among the novel optical spectroscopic techniques available up to date, surface-enhanced Raman scattering (SERS) spectroscopy and flow linear dichroism (FLD) techniques are characterized by high sensitivity and selectivity of analysis of individual components within the supramolecular complexes. SERS spectroscopy proved to be a powerful technique for selective analysis of the structure of low-molecular-weight ligands (e.g., antitumor drug) in complexes with a high-molecular-weight (DNA, protein) target.[2,3] Compared to the other techniques, SERS spectroscopy allows us to detect signals from the individual molecular groups at extremely low (down to 10^{-8} M) concentrations.[4−6] Molecular models of interactions of some antitumor drugs and DNA have been proposed and were found to be consistent with NMR and/or X-ray data.[4−7]

The linear dichroism technique enables us to determine the drug's orientation relative to the axis of oriented in the flow (FLD) or with the electric field (ELD) DNA or protein matrix and was found to be extremely sensitive to the changes of degree of DNA molecule orientation induced by intercalated drugs. So both drug and DNA alterations induced by their interactions may be followed by linear dichroism techniques.[8, 9]

Most of the antitumor drugs are presumed to induce their effects at the DNA level.[10] Some of them are known to be effective inhibitors of the topoisomerases, intranuclear enzymes participating in all aspects of cellular replication and transcription machinery.[1,11] The enzymes' poisons kill cells by trapping so-called cleavable complexes between the DNA and topoisomerases. The chemical structure of poisons and mode of their interactions with DNA determine its ability to interfere with topoisomerases in the cleavage complexes. Structural analysis and identification of functional molecular determinants of individual partners of these supramolecular complexes are quite complicated and require careful selection of appropriate and self-complementary physicochemical techniques but present an

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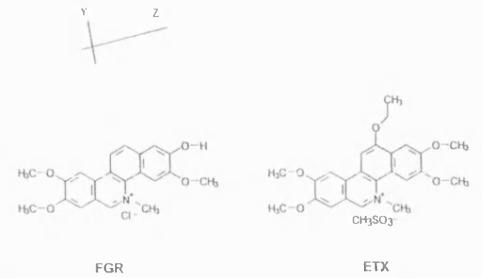

Figure 1. Chemical structures of fagaronine (FGR) and ethoxidine (ETX).

extremely important domain of research, being vital for an adequate new drug's design.[1]

Recently, we have applied a combined SERS, Raman, CD, and biochemical approach to study molecular mechanisms involved in the formation of binary and ternary complexes between antitumor drugs and their targets (DNA and topoisomerases). Molecular interactions within the complexes of well-known (doxorubicine, aclacinomycin, saintopin, netropsins) and new (intoplicine, camptothecin derivatives) compounds that are just in clinics or have potential antitumor activities have been studied.[2,4−6,12]

This paper describes the SERS and FLD analysis of DNA binding by fagaronine (FGR), a natural antileukemic alkaloid and potent differentiation inducer of various hematopoietic cell lines, and by its even more biologically active derivative ethoxidine (ETX). FGR is known to be a potent double inhibitor of DNA topoisomerases I and II, stabilizing in vitro cleavable ternary complexes between the enzymes and their DNA substrates.[13,14] It was shown that the drug is a DNA intercalator,[15] but the mode of its DNA binding is not known and its structural determinants playing a key role in interactions remain to be defined. Cooperative use of SERS and FLD techniques for the study of FGR/DNA and ETX/DNA complexes enabled us to address the following questions. (i) What is the orientation of the drug's chromophores within the drug/DNA complexes? (ii) What are the molecular groups responsible for the drug/DNA recognition and formation of complexes?

We have found that the OH group of FGR participates directly in the drug/DNA binding. According to our model, FGR intercalates within the DNA bases so that its OH group is directed toward the DNA minor groove and so that quaternary N^+ (Figure 1) plays the role of anchor for stabilizing this interaction. ETX is proposed to intercalate within the DNA in a way that its OCH_2CH_3 moiety (Figure 1) extends into the DNA minor groove and probably plays a key role in the disturbance of DNA recognition by the topoisomerases that attack their DNA substrates from the side of the minor groove.

Materials and Methods

Chemicals. FGR (NSC 157995), provided by Dr. E. Couillerot, was extracted from the roots of *Fagara xanthoxyloides* Lam. (Rutaceae) as described in ref 16. ETX was synthesized by one of us, and the details of the protocol will be published elsewhere. Both compounds were prepared in a 1 mM stock solution in methanol.

CT DNA was purchased from Sigma and dissolved in potassium-buffered saline (PBS) to 5 mg/mL.

Drug/DNA complexes were prepared by mixing the drug stock solutions with the DNA solution in PBS in order to obtain a final drug/DNA bp ratio (r) from 0.005 to 0.8.

Raman and SERS Spectroscopy. Raman and SERS spectra were recorded with a PHO (CODERG) spectrometer with a double monochromator in the frequency range 300−1800 cm^{-1}. An Ar^+ ion laser (Coherent Radiation, model Innova 2020) operating at 80 mW power at 457.9 nm (for FGR) or at 488 nm (for ETX) wavelength was used for spectra excitation. For Raman spectra the accumulation of 30 independent scans with time averaging was used to improve the signal-to-noise ratio. SERS spectra were recorded for 1 scan with a 1 s time constant. Silver hydrosol was prepared according to the protocols published before.[4−6] The concentrations of the drugs used for Raman spectroscopy were 5 mM, and the SERS spectra were recorded with the 10^{-5}−10^{-8} M concentrations for free drugs and 10^{-4}−10^{-5} M for drug/DNA complexes.

UV/Visible and Flow Linear Spectroscopy. UV/vis spectra were recorded with a Philips PU8720 UV/vis scanning spectrophotometer.

FLD spectra of DNA and drug/DNA complexes in the region 220−450 nm were recorded with a Jobin Yvon, Mark III dichrograph equipped with a self-made achromatic $\lambda/4$ device to transform circular polarized light to a linear one. The self-made flow cell with an optical length of ca. 0.5 mm and a volume of 200 μL was used for orientation of DNA in the flow. The details of the FLD measurements were published elsewhere.[17]

The linear dichroism ΔA is defined as the difference at a given wavelength between the absorbance for light polarized parallel ($A_{\parallel}$) and perpendicular ($A_{\perp}$) to the flow. The reduced linear dichroism is

$$\mathrm{LD}_r = \Delta A/A = (A_{\parallel} - A_{\perp})/A$$

where A is the isotropic absorbance of the sample.

The angle β between the transition moment of the dye chromophore and the orientation axis of the DNA molecules was calculated from the measured ratios of the reduced linear dichroism for the bases and for the drugs:

$$(\Delta A/A)_{\mathrm{drug}}/(\Delta A/A)_{\mathrm{DNA}} = (3\cos^2\beta - 1)/(3\cos^2\alpha - 1) \quad (1)$$

where $\alpha = 86°$ is the angle between transition moment of the bases and the orientation axis of the DNA molecule.[8]

Results

UV/Visible Spectra of FGR and ETX and Their Complexes with DNA. UV/vis spectra of FGR and ETX show two groups of bands (Figure 2): one in the region 380−420 nm and the second, which is more intense, in the region 270−350 nm. Both groups of the bands correspond to the $\pi \rightarrow \pi^*$ electronic transitions of the conjugated chromophore rings system of types L_b and L_a, respectively.[18] The L_a transition moment is directed along the Z-axis, whereas L_b lies along the Y-axis of the chromophore molecule (Figure 1). Complexation of the drugs with the DNA induces an increase of intensity of the L_b group of bands in the absorption spectra of FGR as well as ETX (Figure 2).

FLD of FGR/DNA and ETX/DNA Complexes. Figure 3 shows reduced linear dichroism as a function of [DNA]/[drug] ratios for FGR and ETX molecules. Both curves were found to possess a maximum. For FGR the highest value of the reduced linear dichroism is observed for a [DNA]/[drug] ratio of about 2.0, whereas for ETX this value corresponds to [DNA]/[drug]

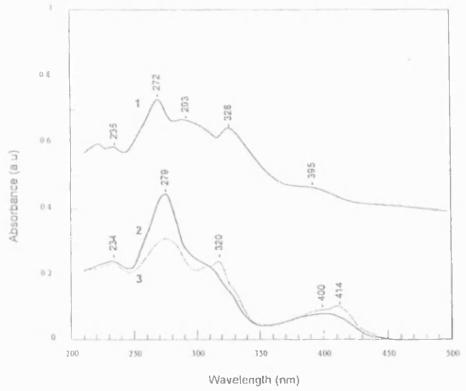

Figure 2. UV/vis spectra of FGR (1), ETX (2), and ETX/DNA complex (3). Drugs concentrations are 10 μM. DNA concentration is 100 μM (bp).

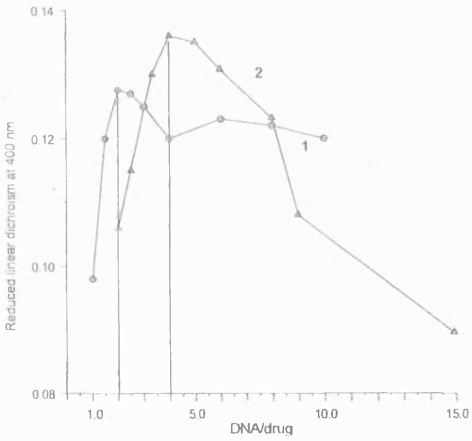

Figure 3. Dependence of the reduced linear dichroism (LD_r) for FGR (1) and ETX (2) on the [DNA]/[drug] ratio. LD_r was measured at 400 nm. DNA concentration (bp) is 10 μM.

ratio at ca. 4.0. These positions of maxima on the reduced linear dichroism curves indicate the points of DNA "saturation" with the drugs. So FGR and ETX interact with the DNA in a binding stoichiometry of 1 drug per ca. 2.0 bp of DNA and 1 drug per 4.0 bp of DNA, respectively.

The FLD technique enables us to determine the relative orientation of the plane of the drug chromophore to the plane of DNA bases; the linear dichroism of intercalators is known to be negative, whereas that of the minor groove binders is positive.[9] FLD signals from FGR and ETX bound to DNA at "saturation" ratios ([DNA]/[drug] = 2.0 and 4.0, respectively) were found to be negative in both L_a and L_b regions of electronic transitions. In other words, both compounds were found to be typical major groove intercalators with their chromophore's planes rather perpendicular to the flow.

Then eq 1 was employed to calculate the angle between the L_b electronic transition of the drug's chromophore and the axis of orientation of the DNA molecule. These angles were found to be ca. 73 ± 5° for FGR and 79 ± 5° for ETX. Owing to the strong absorption of the DNA base pairs in the region of the L_a

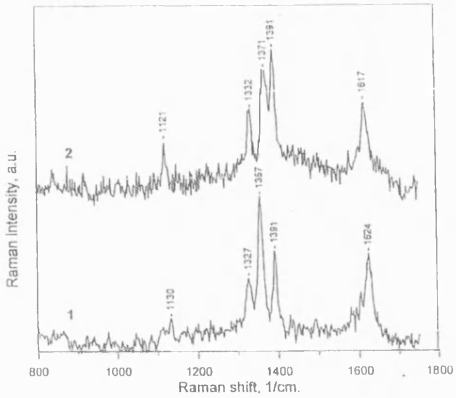

Figure 4. Raman spectra of FGR (1) and ETX (2) in H_2O. λ_{exc} is 457.9 nm for FGR and 488 nm for ETX. Laser power is 50 mW. Drugs concentrations are 5 mM.

electronic transitions of the drugs, we failed to determine the exact angle between this electronic transition moment of the drug's chromophore and the DNA orientation axis. By subtracting the FLD spectrum of DNA from the FLD spectrum of the bound drug, we obtained an approximate value of 80−85°. Anyway, the plane of the drug's chromophore was found to be nearly perpendicular to the DNA orientation axis, that is, almost parallel to the plane of the DNA bases.

Raman Spectra of FGR and ETX. Raman spectra of FGR and ETX are characterized as a "preresonance" recorded with excitation in the region of the drug absorption (Figure 2). The spectra of FGR and ETX recorded at pH 7.5 appeared to be quite similar (Figure 4) with the four intense bands at ca. 1620, 1391, 1360−1370, and 1330 cm^{-1} and a band at 1120−1130 cm^{-1} with moderate intensity. The position and relative intensities of the bands were found to be unchanged at pH 4.0−11.0, and no spectral effects of deuteration was observed (data not shown). So these bands represent skeletal vibrations of the conjugated ring system with probable contribution of $\delta(CH_3)$ motions.

SERS Spectra of FGR and ETX. *General Characteristics.* When the region of chromophore absorption is excited, the SERS spectra of the drugs arise because of surface enhancement itself coupled with the resonance enhancement.[2,3] As a result, an extremely high sensitivity for SERS of FGR and ETX was observed, so we managed to record spectra at very low limits (down to 10^{-9}−10^{-10} M). At the same time, the SERS spectrum of FGR was found to be about 100-fold more intense than that of ETX. There is no direct evidence that the resonance contribution to the overall enhancement must be different for each of these compounds because electronic structures of FGR and ETX are very much the same as seen from their UV/vis spectra (Figure 2). Therefore, the peculiarities in surface enhancement of the Raman signal must account for the differences observed for the SERS spectra of FGR and ETX. It is known that the "chemical" (or short- range) enhancement plays the major role in SERS on silver hydrosols.[19] In other words, only compounds with a high ability to be adsorbed by the hydrosol may exhibit strong Raman enhancement. Adsorptivity is affected by the presence of charged and/or hydrophobic (hydrophilic) groups.[3] Therefore, we propose that the difference in the chemical structures of FGR and ETX should explain the higher adsorption ability of FGR and, consequently, higher

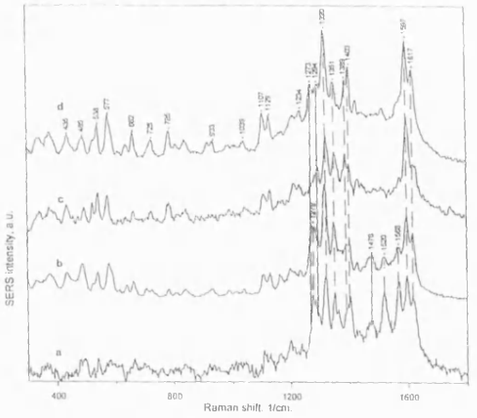

Figure 5. SERS spectra of FGR at pH 11.0 (a), 7.5 (b), 4 (c) and in a complex with DNA (d) at pH 7.5. Drug concentration is 1 μM. DNA concentration (bp) is 0.1 mM. λ_{exc} = 488 nm. Laser power is 25 mW.

Raman enhancement factor. On the other hand, if the OH group of FGR determines its surface enhancement, any changes in the environment or interactions of this group (e.g., hydrogen bonding) would influence the SERS intensity of FGR.

The SERS spectra contain four strong bands (Figures 5 and 6), which have their counterparts in the Raman spectrum (ca. 1620, 1400, 1350, and 1320 cm^{-1} for FGR and ca. 1610, 1385, 1370, and 1330 cm^{-1} for ETX). These can be attributed to skeletal vibrations of the conjugated ring system. The band at 1380−1400 cm^{-1} may be also contributed from $\delta(CH_3)$ and the band at 1320−1330 cm^{-1} from C−C−H motions.[20] In Wilson's notation of vibration modes of benzene, the bands in the region 1590−1610 cm^{-1} are assigned to ν_{8a} and ν_{8b} vibrations.[21] The relative intensities of these bands are known to be extremely sensitive to the mass of the chromophore substituent group[20] and its interactions with the ions.[6] Figures 5 and 6 show that the SERS band of FGR at ca. 1595 cm^{-1} is more intense than that at ca. 1615 cm^{-1}, whereas for ETX the band at ca. 1611 cm^{-1} is more intense than 1597 cm^{-1}. This difference correlates with the difference in the structures of FGR and ETX; the OH group of FGR is replaced by the heavier OCH_3 group in ETX (Figure 1). Moreover, an additional massive OCH_2CH_3 group is presented in the ETX molecule. Since these structural differences of FGR and ETX may be easily detected by SERS spectroscopy, we may expect that any changes in the microenvironment of the OH group of FGR or any interactions of this group with the other moieties should be elicited in the SERS spectrum as a change in the relative intensity of the bands corresponding to ν_{8a} and ν_{8b} vibrations.

In addition to the bands mentioned above, there are some features in the region 400−600 cm^{-1} of the SERS spectra of FGR and ETX that may be attributed to δ(C−C−O) and δ-(C−O−C) vibrations: the bands in the region 1100−1140 cm^{-1}, likely interpreted as due to ν(C−O−CH_3) and ν(C−C−O) motion, and the several bands in the region 1200−1600 cm^{-1} typically assigned to the normal vibration of benzene derivatives.[20,21]

Effect of pH on the SERS Spectra. To identify additional spectral indicators for the OH group microenvironment of FGR and to determine the contribution of N^+ (Figure 1) to the vibrational structure of FGR and ETX, we have analyzed the pH dependence of the SERS spectra. At basic pH, the OH group of FGR is expected to be deprotonated, whereas at acidic pH some perturbations in the microenvironment of N^+ are assumed.

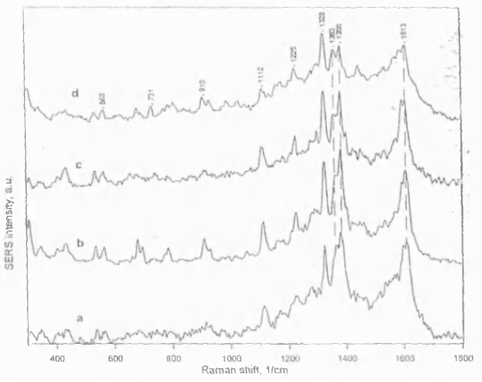

Figure 6. SERS spectra of ETX at pH 11.0 (a), 7.5 (b), 4 (c) and in a complex with DNA (d) at pH 7.5. Drug concentration is 10 μM. DNA concentration (bp) is 0.5 mM. λ_{exc} = 488 nm. Laser power is 25 mW.

First, it should be noted that the overall intensity of the SERS spectra at pH different from neutral is significantly lower than those at pH ca. 7.5. This may be explained by partial degradation of hydrosol at basic and acidic pH that leads to lower SERS enhancement factors.[3] For the ETX molecule (which does not possess the OH group; see Figure 1) the "pure" effect of the N^+ environment on the SERS spectrum may be determined. The SERS spectrum of ETX, recorded at pH 11.0 (Figure 6a), shows slight changes compared to its spectrum at pH ca. 7.5 (Figure 6b). The only detectable difference is a relative decrease of the 1370 cm^{-1} band at neutral pH. At pH close to 4.0 a further loss of this band occurs compared to the band at ca. 1330 cm^{-1}. Moreover, some decrease of the 1380 cm^{-1} band and an increase of the band at ca. 1610 cm^{-1} are also observed. The bands sensitive to pH were attributed to skeletal vibrations of the conjugated ring system with contribution of the $\delta(CH_3)$ motion. Therefore, we may conclude that pH and, subsequently, the environment of N^+ slightly affect the vibrational structure of ETX.

FGR comprises two pH-dependent structural elements: OH group and N^+ (Figure 1). Having established pH-induced spectral changes arising from the variation of the environment of N^+ (in case of ETX), we may interpret all other changes in the SERS spectrum of FGR in terms of pH-dependent modifications of the OH group. Deprotonation of the OH group of FGR at basic pH induces significant changes in its SERS spectrum (curves a and b of Figure 5). First of all, there are two bands at 1520 and 1568 cm^{-1}, relative intensities of which are strongly increased at pH ca. 11.0. In addition, there is a redistribution of the relative intensities of the bands in the region 1250−1450 cm^{-1} compared to the spectrum at neutral pH. At acidic pH we find a total disappearance of the bands 1476, 1520, and 1568 cm^{-1}, a shift of the band at ca. 1279 cm^{-1} to 1294 cm^{-1} with a simultaneous decrease of their relative intensities and increase of the band at ca. 1600 cm^{-1} (Figure 5c). In addition to these changes, all the SERS bands were found to decrease at pH 11.0 versus pH 4.0. We suppose that the bands at 1520 and 1568 cm^{-1} should be assigned to C=C vibrations of the aromatic ring with possible contribution of the N^+=C vibration to 1568 cm^{-1}, whereas the band in the 1270−1295 cm^{-1} region most probably corresponds to the ν(C−O) vibration.[20] Although these

bands cannot be attributed directly to the vibrations of the OH group, they undoubtedly depend on the microenvironment of this group.

The following spectral indicators of the OH group microenvironment in FGR have been characterized. The first is the overall intensity of the SERS spectrum revealed from the comparison of FGR and ETX spectra. The second is the high sensitivity of relative intensities of the ν_{8a} and ν_{8b} vibrations to any interactions of the OH group. Finally, we have revealed some other pH-dependent bands sensitive to the protonation state of the OH group. We can propose therefore the following correlation between the SERS spectrum and the environment of the OH group. Deprotonated at basic pH, the OH group is characterized by strong bands at 1520 and 1568 cm^{-1} and a relatively strong band at ca. 1279 cm^{-1}. At nearly neutral pH 7.5, some fraction of the OH groups are still deprotonated. Therefore, the bands at 1520 and 1568 cm^{-1} as well as at 1279 cm^{-1} are observed but weaker than at basic pH. At acidic pH the 1520 and 1569 cm^{-1} bands completely disappear, the band at ca. 1278 cm^{-1} shifts to 1294 cm^{-1}, and, finally, a significant increase of the band corresponding to ν_{8a} is observed. It should be noted that the latter change might arise not only from interactions of the OH group but also from the O−CH_3 group environment, since the same effect was observed for ETX.

SERS Spectra of FGR/DNA and ETX/DNA Complexes. The SERS spectra of FGR/DNA (Figure 5d) and ETX/DNA (Figure 6d) complexes were recorded at different [drug]/[DNA] ratios (from 0.001 to 0.5). It is known[2−7] that the intensity of the SERS signal for intercalating agents (such as adriamycin, saintopin, intoplicine) decreases significantly under complexation with DNA. In drug/DNA complexes the drug is buried within the DNA duplex so that it is kept away from the surface, and thereby, the SERS sufficiently decreases.

The same effect was observed for FGR and ETX. In the complex with DNA, the SERS signal was found to be 10−20 times weaker than for the free drug. Using this fact, we have determined the DNA/drug association constant for FGR to be ca. 3×10^5 M^{-1}. This value is close to that determined previously by viscosimetric measurements.[13] Therefore, we conclude that interactions between the drug and DNA are poorly, if at all, affected by the surface and that the information obtained with SERS spectroscopy may be compared and correlated with that obtained with other techniques (UV/vis, CD, FLD). The same conclusion has been drawn before for a number of other anticancer agents.[2−7]

To deduce spectral changes induced by complexation with DNA, the spectra of free drugs were compared to those of the [DNA]/[drug] complex at a 200:1 ratio. Under these conditions all the drug molecules are ensured to participate in the complex and no contribution of the free drugs is present. Comparing the SERS spectrum of the ETX/DNA complex with that of the free drug (Figure 6), we find the following spectral differences. The bands in the region 1370−1400 cm^{-1} decrease in relation to the 1328 cm^{-1} band; the intensity of the band at 1113 cm^{-1} is relatively lower for the complex than for the free drug, and finally, some intensity alterations were observed in the region 1580−1620 cm^{-1}.

As was indicated above, the bands in the region 1370−1400 cm^{-1} correspond to skeletal vibrations of the conjugated ring with some contribution of $\delta(CH_3)$ motions. Therefore, these bands hardly provide particular information concerning interactions of individual molecular groups of ETX. Any change in the state of these groups may lead to an alteration of the intensity of skeletal vibrations. Nevertheless, this spectral range was found to be sensitive to the modification of the N^+ environment. Thus, we have indications that N^+ is involved in the interaction with DNA. Moreover, the band at 1113 cm^{-1} (assigned to the ν(C−O−CH_3) vibration) clearly indicates that the exterior group O−CH_3 is involved in the interaction with DNA. Finally, as was shown above, bands in the 1580−1620 cm^{-1} region may also serve as indicators of interaction of the O−CH_3 group.

The spectral changes observed for SERS of FGR/DNA complexes (Figure 5) are more significant than those for ETX. The differences include a decrease or even disappearance of the bands at 1476, 1520, and 1570 cm^{-1}, several modifications of band intensities in the 1330−1420 and 1590−1610 cm^{-1} regions, a relative increase of the bands at 1100−1130 cm^{-1}, and finally, a splitting of the 1278 cm^{-1} band into two bands at 1292 and 1272 cm^{-1}. These changes are very similar to those observed for SERS spectra of the free drug at acidic and neutral pH and may be attributed to the changes of the environment of N^+ and interactions of OH and O−CH_3 groups. Therefore, we suppose that the effect of DNA on the structure of the drugs occurs through interactions between the OH, O−CH_3, and N^+ groups of the drug with the DNA.

The band at 1278 cm^{-1} corresponds to the ν(C−O) vibration and was shown to be sensitive to the formation of the H bond with participation of OH groups of various chromophores.[7,22] The presence of the two peaks at the expected position of this band in the spectrum of the drug/DNA complex may be explained as follows. As we have established, a significant number of OH groups at neutral pH still exist in deprotonated form. Therefore, we may observe the same position of this band at neutral as at basic pH. Upon complexation with DNA some bond(s) between DNA and the OH group appeared. These bonds are most likely H bonds, but we are not able to distinguish if the oxygen serves as a donor or as an acceptor of proton. It is reasonable to suppose that the oxygen in the deprotonated OH group is an acceptor of a proton, while the oxygen of the protonated OH group may be either an acceptor or a donor. Therefore, we propose that in the SERS spectrum of the drug/DNA complex the bands at 1272 and 1292 cm^{-1} correspond to vibrations of the OH group participating in H-bonding as an acceptor and/or a donor of proton.

Discussion

There are two principal "extreme" modes of anticancer drug interactions with DNA: minor groove binding and intercalation. The only exception is specific DNA topoisomerase I inhibitor camptothecin, exhibiting the extremely low affinity to DNA or topoisomerase I alone but exhibiting its anticancer activity by stabilizing the ternary cleavage complex between DNA and topoisomerase I enzyme and thus preventing the substrate's religation.[23,24]

These two "extreme" modes of DNA binding by drugs were found to be distinguishable using linear dichroism spectroscopy.[9] The intercalators exhibit a negative signal in the LD spectrum, whereas the minor groove binders give rise to a positive signal. The most usual "mixed mode" of DNA interaction of agents induces normally the superposition of the features characteristic for the two "extreme" modes.

In the present study we have demonstrated that FGR and ETX induce negative LD signals. Moreover, precise calculations of the angle between the transition moment of the drug and the DNA orientation axis show that the planes of the drug's chromophores are nearly parallel to the plane of the nucleotide bases. Therefore, we may conclude that both compounds, FGR and ETX, are intercalators. The conclusion concerning FGR

supports the result obtained before by measurements of the DNA length increase induced by FGR/DNA interactions.[13]

In addition to traditional methods providing information on DNA/drug interactions, SERS spectroscopy allows the selective detection and study of molecular interactions of individual molecular groups of drugs. To date, this method was applied to the analysis of a number of anticancer drugs and their interactions with DNA.[2-7] In the case of FGR and ETX, SERS appeared to be an extremely sensitive and selective technique, owing to tremendous enhancement of Raman scattering from these molecules. The SERS spectroscopy reveals pronounced differences in molecular interactions of FGR and ETX with DNA. These differences were explained in terms of the chemical nature of the substituent groups of the drugs. The OH group of FGR was found to play a key role in the drug's interactions with DNA. In addition, the N^+ and OCH_3 moieties (Figure 1) should be involved in DNA binding. Replacement of the OH group by OCH_3 and the presence of an additional OCH_2CH_3 group in an ETX molecule should lead to redistribution of electric charge in the chromophore conjugated system. As a result, the orientation of the total dipole moment of the molecule should be different for FGR and ETX. Therefore, one may suggest that, upon intercalation, orientation of the ETX molecule with respect to DNA bases is quite different from that of FGR.

In the previous study of FGR and its complexes with DNA by UV/vis spectroscopy it was proposed that the quaternary cation (N^+) may serve as an anchor that binds the negatively charged phosphate group of the backbone of double-stranded nucleic acids.[15] Hereupon initial interaction a more hydrophobic region of the molecule could intercalate between the stacked base pairs.

In the studies of protoberberine analogues whose chemical structures are rather similar to those of FGR and ETX, the so-called "mixed-mode" DNA binding model was presented.[25] This model suggests that a portion of the ligand intercalates into the double helix, while the nonintercalated portion of the molecule protrudes into the minor groove of the host duplex, where it becomes available for interactions with the atoms lining the floor and/or walls of the minor groove.

This "mixed-mode" DNA-binding model seems very suitable for FGR and ETX molecules. Indeed, the quaternary cation may serve as an anchor that binds the negatively charged phosphate backbone of the double-stranded nucleic acids, and the molecule could intercalate between the stacked base pairs. However, a significant part of the molecule enters the DNA minor groove. We suppose that FGR is oriented so that its OH group is rather directed to minor groove, whereas the OCH_2CH_3 group of ETX protrudes into the minor groove where it becomes accessible for interactions with the DNA-binding intracellular enzymes.

FGR is known to be a potent inhibitor of DNA topos I and II, stabilizing the cleavable complex between the enzyme and its DNA substrate.[13,14] ETX, a synthetic derivative of FGR, seems to be also an effective double inhibitor of topos[26] with the even higher cytotoxicity on the cellular level. This paper presents the first comparative study of the molecular interactions of these two antitumor agents with the DNA. The differences of the modes of DNA binding by FGR and ETX, especially in the terms of their molecular interactions within the DNA minor group (minor-groove-directed position of the OH group of FGR and minor-groove occupation with the spacious OCH_2CH_3 group of ETX), should obviously modulate the topos inhibitory effect of these drugs. The next stage of our work will concern the comparative analysis of molecular interactions of FGR and ETX within the ternary cleavable complexes with the DNA and topos. This work should provide us with the information enabling us to correlate the in vitro results with the cytotoxicity effects exhibited by these drugs at the cellular level.

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Glossary

SERS	surface-enhanced Raman scattering
FLD	flow linear dichroism
LD	linear dichroism
CD	circular dichroism
FGR	fagaronine
ETX	ethoxidine (12-alkoxy-benzo[*c*]phénanthridine)
topo (s)	topoisomerase (s)
PBS	potassium-buffered saline
sc	supercoiled

References and Notes

(1) Capranico, G.; Zunino, F. *Curr. Pharm. Des.* **1995**, *1*, 1.

(2) Nabiev, I. R.; Sokolov, K. V.; Manfait, M. *Biomolecular Spectroscopy*; Wiley: London, 1993; Chapter 7, pp 267−338.

(3) Nabiev, I.; Chourpa, I.; Manfait, M. *J. Raman Spectrosc.* **1994**, *25*, 13.

(4) Nabiev, I.; Chourpa, I.; Riou, J.-F.; Nguyen, C. H.; Lavelle, F.; Manfait, M. *Biochemistry* **1994**, *33*, 9013.

(5) Nabiev, I.; Chourpa, I.; Manfait, M. *J. Phys. Chem.* **1994**, *98*, 1344.

(6) Feofanov, A.; Ianoul, A.; Oleinikov, V.; Gromov, S.; Fedorova, O.; Alfimov, M.; Nabiev, I. *J. Phys. Chem. B* **1997**, *101*, 4077.

(7) Smulevich, G.; Feis, A. *J. Phys. Chem.* **1986**, *90*, 6388.

(8) Matsuoka, Y.; Nordén, B. *Biopolymers* **1982**, *21*, 2433.

(9) Bailly, C.; Henichart, J. P.; Colson, P.; Houssier, C. *J. Mol. Recognit.* **1992**, *5*, 155.

(10) Monnot, M.; Mauffret, O.; Simon, V.; Lescot, E.; Psaume, B.; Saucier, J. M.; Charra, M.; Belehradek, J.; Fermandjian, S. *J. Biol. Chem.* **1991**, *266*, 1820.

(11) Wang, J. C. *Annu. Rev. Biochem.* **1996**, *65*, 635.

(12) Fleury, F.; Ianoul, A.; Kryukov, E.; Sukhanova, A.; Kudelina, I.; Wynne-Jones, A.; Bronstein, I. B.; Maizieres, M.; Berjot, M.; Dodson, G. G.; Wilkinson, A. J.; Holden, J. A.; Feofanov, A. V.; Alix, A. J. P.; Jardillier, J. C.; Nabiev, I. *Biochemistry* **1998**, *37*, 14630.

(13) Larsen, A. K.; Grondard, L.; Couprie, J.; Desoize, B.; Comoe, L.; Jardillier, J.-C.; Riou, J.-F. *Biochem. Pharmacol.* **1993**, *46*, 1403.

(14) Wang, L. K.; Johnson, R. K.; Hecht, S. M. *Chem. Res. Toxicol.* **1993**, *6*, 813.

(15) Pezutto, J. M.; Antosiak, S. K.; Messmer, W. M.; Slaytor, M. B.; Honig, G. R. *Chem. Biol. Interact.* **1983**, *43*, 323.

(16) Messmer, W. M.; Tin-Wa, M.; Fong, H. H. S.; Bevelle, C.; Farnsworth, N. R.; Abraham, D. J.; Trojavek, J. *J. Pharm. Sci.* **1972**, *61*, 1858.

(17) Bronstein, I.; Wynne-Jones, A.; Sukhanova, A.; Fleury, F.; Ianoul, A.; Holden, J.; Alix, A. J. P.; Dodson, G.; Jardillier, J. C.; Nabiev, I.; Wilkinson, A. J. *Anticancer Res.*, in press.

(18) Klevens, J.; Platt, M. J. *Chem. Phys.* **1949**, *17*, 470.

(19) Hildebrandt, P.; Stockburger, M. *J. Phys. Chem.* **1984**, *88*, 5935.

(20) Schrader, B. *Raman/Infrared Atlas of Organic Compounds*; VCH: Weinheim, Germany, 1989.

(21) Wilson, E. B. *Phys. Rev.* **1934**, *45*, 706.

(22) Nonaka, Y.; Tsuboi, M.; Nakamoto, K. *J. Raman. Spectrosc.* **1990**, *21*, 133.

(23) Gupta, M.; Fujimori, A.; Pommier, Y. *Biochim. Biophys. Acta* **1995**, *1262*, 1.

(24) Liu, L. F., Ed. *Adv. Pharmacol.* **1994**, *29B*, 305.

(25) Pilch, D. S.; Yu, C.; Makhley, D.; La Voie, E. J.; Srinivasan, A. R.; Olson, W. K.; Sauers, R. R.; Breslauer, K. J.; Geacintov, N. E.; Liu, L. L. *Biochemistry* **1997**, *36*, 12542.

(26) Sukhanova, A.; Fleury, F.; Devy, J.; Ianoul, A.; Duval, O.; Alix, A. J. P.; Jardillier, J. C.; Nabiev, I. *Mol. Pharmacol.*, in press.

J. Med. Chem. **2000**, *43*, 3257−3266

DNA Binding, Solubility, and Partitioning Characteristics of Extended Lexitropsins

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Four new ligands that bind to the minor groove of DNA have been designed, synthesized, and evaluated by DNA footprinting. Two of the ligands are polyamides containing central regions with five or six *N*-methylpyrrole units, conferring hydrophobicity and good binding affinity but without retaining the correct spacing for hydrogen bonding in the base of the minor groove. The two remaining ligands have central regions which are head-to-head-linked polyamides, in which the linker is designed to improve the phasing of hydrogen bonding of the ligand with the floor of the minor groove. The highest affinity was obtained with the two polypyrroles without headgroup spacers, indicating that H-bond phasing is secondary in determining affinity compared to the major hydrophobic driving force. With a dimethylaminoalkyl group, representing a moiety with modest base strength, at both ends, water solubility is good and pH-partition theory predicts that penetration through lipid membranes will be enhanced, compared to strongly basic amidine analogues of the alkaloid precursors. All four compounds bind to DNA, with strong selectivity for AT sequences but some tolerance of GC base pairs and subtle individual preferences. The data show that very high affinities can be anticipated for future compounds in this series, but drug design must take account of overall physicochemical properties as well as the details of hydrogen bonding between ligands and the floor of the minor groove.

Introduction

The attractions of drug design where the ligand is directed at the primary coding sequences of DNA are many. Such a ligand, should it be sufficiently sequence-selective, would offer the prospect of gene control in diseases where genetic malfunction is the primary cause, such as cancer. In addition, there are exciting possibilities for the treatment of parasitic diseases, where the DNA possesses sequences which do not occur in the host.

Sequence selectivity is not a major feature of the early type of intercalating ligands, such as ethidium, but much attention has been devoted in recent years to the development of 'lexitropsins', which bind to the minor groove of DNA and thus have a greater chance of reading the information which is provided by the base-pair sequence as part of the usual process of transcription. It was found quite early in these studies that the natural polypyrroles, such as netropsin (**1**) and distamycin (**2**), showed almost total selectivity for AT rather than GC sequences.[1] Very recently, it has been shown that these compounds show a degree of selectivity for some specific AT sequences over others.[2,3]

An important advance was made in ligand design[4] when the *N*-methylpyrrole units of the natural products were replaced with other five-membered heterocyclic rings, in particular imidazole, which changed the sequence-binding preference from AT to GC. A combination of these units offers the possibility of designing molecules to bind preferentially to any given double-stranded DNA sequence. Additionally, it is necessary to extend the length of the molecule to read considerably more than the four base pairs which bind the natural products, if a useful level of selectivity is to be achieved,[5] which may require the use, for example, of bifunctional linkers[6] to join chains of amide-bonded heterocycles. A significant variation on this approach allows the chain to double back on itself.[7] Such a 'hairpin' structure fills the minor groove and reads the sequence information more efficiently than a single chain but requires the construction of a considerably larger molecule, up to double the length of the sequence which is being read.

The central part of the ligand must be hydrophobic, since the main driving force for binding is the expulsion of water from the minor groove, itself a largely hydrophobic environment with a capacity for hydrogen bonding at the bottom of the groove. A recent thermodynamic study[8] has shown that binding in the minor groove of a DNA dodecamer by Hoechst 33258 is dominated by hydrophobic forces; clearly, new ligands must have large nonpolar regions if they are to show high affinity. A major concern in drug design, therefore, is the water

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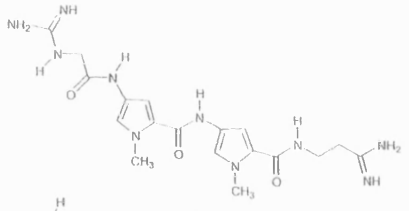
solubility of a ligand composed largely of nonpolar subunits and, if this is overcome by the use of more than one basic end group, the effect that this will have on the ability of the molecule to penetrate lipid membranes by passive diffusion.



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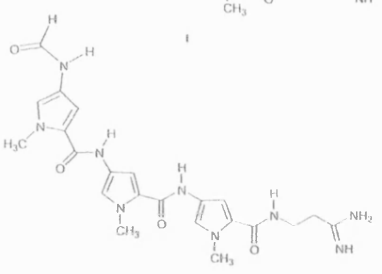

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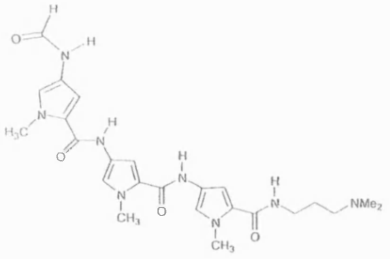

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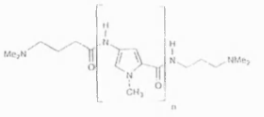
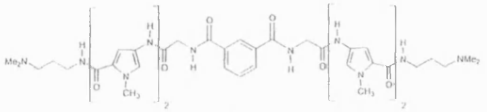
To test these concepts, we have synthesized four new ligands **4**–**7**, which combine building blocks known to give good DNA binding, and have correlated their DNA binding with their calculated partitioning properties. Ligands **4** and **5** are extended polyamides, with long chains of nonpolar *N*-methylpyrroles rendered more soluble by dimethylamino groups at both ends. Ligands **6** and **7** are both head-to-head-linked polypyrroles, with a linker which has been designed to improve H-bonding in the base of the minor groove: in both cases the linker is more hydrophilic than the polypyrrole chains. The dimethylamino end groups of structures **4**–**7** were chosen for three reasons. First, they have been used before[9] and have been shown to be consistent with good DNA binding. Second, the synthetic methods for their incorporation are well-established, and third, we wished to use tertiary bases, which would have lower pK_a values than amidines, guanidines, or secondary bases. This is highly significant when pH-partition theory is applied to these molecules.

The application of pH-partition theory to drugs with a lipophilic structure and one basic group is well-established;[10] it is a combination of the Henderson–



4 n = 5, 5 n = 6



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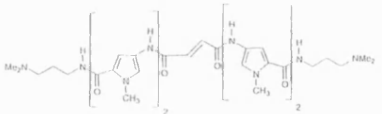

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Hasselbach treatment of the ionization of weak acids and bases with the concept of the partition coefficient (P) between two immiscible solvents, giving eq 1, for a compound with one basic center:

$$D = P/(1 + 10^{(pK_a - pH)}) \qquad (1)$$

In this equation, D is defined as the ratio of the concentration of nonionized material in the organic (lipid) phase to the concentration of the combined ionized and nonionized material in the aqueous phase, at equilibrium. Since, for a given compound P and pK_a are constant, it is possible to plot log D against pH. Where insight is sought into the probable behavior of a molecule in the body, the region of greatest interest is that around pH 7.4, the pH of most body tissues. At pH 7.4 molecules such as **4**–**7** are expected to be doubly charged, in which case the pH-partition behavior is described by a special form of eq 1, taking account of the existence of two basic centers, here assumed to have the same value of pK_a, and a doubly charged species (eq 2). Where the two pK_a values are close, but not identical, this form of the equation can be regarded as an adequate approximation:

$$D = P/(1 + 2(10^{(pK_a - pH)}) + 10^{(2(pK_a - pH))}) \qquad (2)$$

Since the introduction of two charged groups might influence DNA binding, we compared the binding characteristics of compounds **4**–**7** using the footprinting technique.

Discussion

DNA Binding. We used DNase I and hydroxyl radical footprinting to examine the interaction of these ligands with natural and synthetic DNA fragments. With TyrT DNA (Figure 1), a natural DNA fragment which has been widely used in footprinting studies, we found that each of the compounds altered the digestion

TyrT DNA

5′-TCGGGAACCC$_{150}$CCACCACGGG$_{140}$GTATTGCTTT$_{130}$TTACTGGCCT$_{120}$GCT

CCCTTAT$_{110}$CGGGAAGCGG$_{100}$GGCGCATCAT$_{90}$ATCAAATGAC$_{80}$GCGCCGCT

GT$_{70}$AAAGTGTTAC$_{60}$GTTGAGAAAA$_{50}$AATGAACTGG$_{40}$TTGCGTAATT$_{30}$TTCA

TCCGTA$_{20}$ACGGATTAAA$_{10}$GGTAACCGGAA-3′

p(A/T)$_{6-12}$

5′-GATCCGGAATATTCCGGAAATATTTCCGGAAAATATTTTCCGGAAAAAT

ATTTTTCCGGATC-3′

pA$_{3-6}$

5′-GATCGCGTTTTTTCGCGTTTTTCGCGTTTTCGCGTTTCGCGATC-3′

p(A/T)$_{10}$

5′-GATCGCGTTTTTAAAAACGCGTATATATATACGCGAAAAATTTTTCGCG

AATTCGCGATC-3′

p(A/T)$_6$

5′-GATCGCGAAATTTCGCGTATATACGCGTAATTACGCGTTTAAACGCGAA

TTCGCGG-3′

Figure 1. Sequences of the various DNA fragments used in this work. Only the strand bearing the radioactive label (underlined) is shown. TyrT is an *Eco*R1−*Ava*I fragment. The numbering system used for this DNA is the same as that used in previous publications.[11-3] For p(A/T)$_{6-12}$, pA$_{3-6}$, p(A/T)$_{10}$, and p(A/T)$_6$ only the sequence of the insert (which was cloned into the *Bam*H1 site of pUC18) is shown. Every tenth base of the TyrT fragment is numbered for easy reference.

pattern at concentrations of 0.1−3 μM. In each case reductions in cleavage were seen around positions 25, 60, and 80, each of which corresponds to an AT-rich site which has previously been shown to be affected by other minor groove-binding ligands. These patterns were not easy to interpret since DNase I cleavage in the control was very uneven and several regions of poor cleavage in the control were located in the AT-rich regions around positions 25−30 and 45−50, which are expected to be good ligand-binding sites. We therefore repeated the experiments using hydroxyl radicals as the footprinting probe. These produced a much more even ladder of cleavage products in the control, enabling a more precise determination of the ligand-binding sites. Low concentrations of compound **4** (0.3 and 1 μM) produced attenuated cleavage around positions 25−30, 46−50, 61−65, and 81−86. The first two are located toward the 3′-side of long AT tracts (7 and 6 bases, respectively), while the third contains only 4 consecutive AT pairs. However, it may be significant that this lies within an AT-rich region; the 13 AT pairs between positions 59−71 contain only 3 GC pairs. It is possible that this represents ligand binding to a longer site (possibly TGTAAA at 65−70), tolerating a single guanine residue. Each of these footprints was about 6 bases long, compared with 3−4 bases observed with distamycin,[11] confirming the larger ligand-binding site. At higher concentrations (3 μM and above) the footprinting pattern changed and more regions were protected from cleavage. Most notable was a new footprint around position 40 (AACTGGTT). The regions protected around positions 60 and 80 also increased in size and appeared to split into two footprints located on either side of the ones produced at lower concentrations. The pattern produced by 1 μM **5** was similar to that generated by **4** at low concentrations, except that the footprint around position 65 was more clearly defined, while the footprint around position 25 extended over one or two more bases in the 3′ direction, consistent with its larger size. At higher concentrations (3 μM and above) hydroxyl radical cleavage was almost completely abolished. An example of a differential cleavage plot, derived from densitometer scans of the data for 1 μM compound **5** only, is presented in Figure 2A, revealing three distinct regions of protection between positions 20−70. Three clear footprints were also evident with 0.3 μM **7** around positions 25−30, 46−50, and 81−86, as observed with the other ligands, except that there was little protection around position 65. With 1 μM of ligand **7** new sites of protection were evident, corresponding to the pattern produced with higher concentrations of **4**. Compound **7** showed five binding sites between positions 20−77. The first and third (around positions 25 and 45) correspond to long AT tracts, while the latter (around position 65−70) contains the sequence TGTAAA. The other two sites are harder to define rigorously but appear to contain the sequences AACTGGTT (positions 37−44) and TTAGCTT (55−61). The changes with 1 μM **6** were less clear, with only one clear footprint at positions 27−31, with attenuated cleavage around positions 46−50 and 81−86.

These results confirm that the compounds are indeed AT-selective, with larger binding sites than the parent antibiotic distamycin. In addition they suggest that at moderate concentrations these compounds can bind to some sites which contain 1 or 2 GC base pairs and that the various derivatives have subtly different sequence binding properties. We have therefore investigated their interaction with several synthetic DNA fragments containing different length AT tracts and various arrangements of AT base pairs.

Fragment pAAD (not shown) contains 5 (A/T)$_4$ sites, each separated by 6 GC base pairs, and has previously been used to determine AT selectivity of several minor groove-binding ligands.[2] None of compounds **4**−**7** affected either DNase I or hydroxyl radical cleavage of this fragment at concentrations up to 3 μM, though higher concentrations abolished cleavage throughout the fragment. This confirms that, in this fragment, 4 consecutive AT base pairs were not sufficient to constitute a specific ligand-binding site.

Figure 3 shows DNase I cleavage of fragment pA$_{3-6}$ which contains 4 A$_n$/T$_n$ tracts of various lengths (n = 3−6). It can be seen that, with the exception of 3 μM **7**, cleavage of T$_3$ was not affected by the ligands and cleavage at T$_4$ was only attenuated at the highest ligand concentrations. Cleavage of T$_5$ and T$_6$ was inhibited at lower ligand concentrations with T$_6$ as the better site in each case. Both **4** and **7** protected T$_6$ at the lowest concentration (0.3 μM), while T$_5$ was not fully protected below 1 μM. Similarly **5** protected T$_6$ at 1 μM, while T$_5$ required 3 μM. For **6**, T$_6$ was protected at the highest concentration, while the other sites were hardly affected. It therefore appears that the ligands bind best to sites containing 6 consecutive AT base pairs, although there is a significant interaction with sites containing 5 and 4 AT pairs. Hydroxyl radical cleavage patterns with this fragment are not easy to interpret since digestion of the

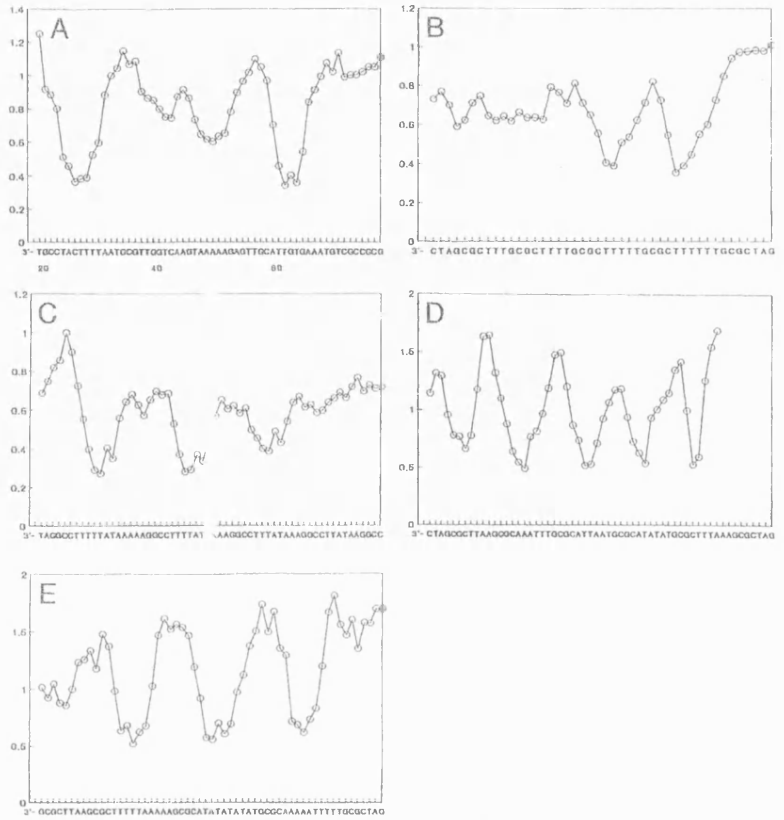


Figure 2. Differential cleavage plots showing the interaction of 1 μM **5** with the 5 different DNA fragments: A, TyrT DNA; B, pA_{3-6}; C, $p(A/T)_{6-12}$; D, $p(A/T)_6$; E, $p(A/T)_{10}$. The data were obtained from hydroxyl radical cleavage patterns and were analyzed as described in the text.

DNA was attenuated in each T_n tract in the absence of the ligand, consistent with previous suggestions that these sequences possess narrower than average minor grooves. A differential cleavage plot comparing the pattern in the presence of 1 μM **5** with that in the control is shown in Figure 2B. With this compound footprints were only produced at T_5 and T_6, each covering 6−7 bases. **6** also produced clear footprints at T_6 and T_5, and the protection at T_4 and T_3 coalesced into a single footprint. Compounds **4** and **7** produced footprints at all the T_n sites, each of which covers 5−6 bases. However, examination of the pattern at T_3 reveals that the apparent protection is far to the 3′-side of the actual site, suggesting that there was something unusual about this interaction.

The results with pA_{3-6} confirmed that the compounds bind best to longer AT tracts and showed that the binding site, as revealed by hydroxyl radicals, covered about 6 base pairs. We therefore extended these studies to a fragment containing longer AT tracts. With hydroxyl radical cleavage of $p(A/T)_{6-12}$, for which the sequence[13] is given in Figure 1, compounds **4** and **5** produced clear footprints covering 6−7 base pairs at the sites containing 8, 10, and 12 base pairs but had little effect on the upper site containing 6 AT base pairs (AATATT) (Figure 3). A differential cleavage plot showing the results for **5** is shown in Figure 2C. In contrast **7** produced footprints at all 4 sites, including AATATT. However the footprint at the longest site was much broader with this ligand affecting 8−9 base pairs. This may be because the ligand does not adopt a unique position within this long AT site but can assume one of several locations each of which does not cover the entire site. Surprisingly 1 μM **6** failed to show appreciable protection at any of the sites. It should be emphasized that each of these hydroxyl radical footprints is different from that produced by the parent antibiotic distamycin, which produces two distinct regions of protection within the 8-, 10-, and 12-base pair sites, consistent with the suggestion that two ligand molecules can simultaneously bind within each site.[13]

Previous studies have shown that, although distamycin and other minor groove-binding ligands can bind to all arrangements of 4 consecutive AT base pairs, some sites are better than others.[2] AATT is a particularly good binding site, while the ligands bind less well to

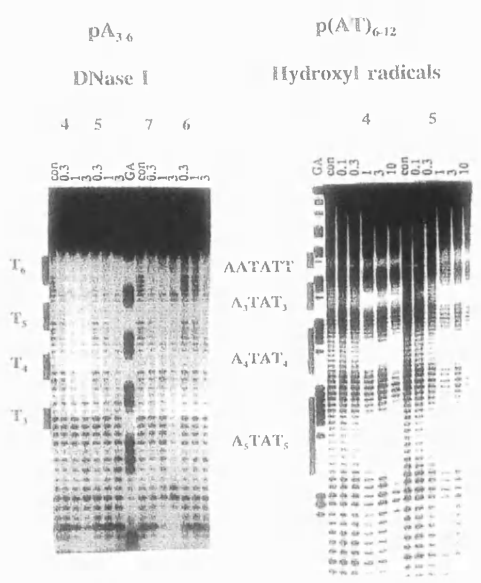


Figure 3. DNase I footprinting patterns for the interaction of compounds **4**–**7** with the fragments derived from pA_{3-6} and $p(AT)_{6-12}$. The concentration of the ligand (μM) is shown at the top of each gel lane. 'Con' indicates the control, in the absence of added ligand. Tracks labeled 'GA' are Maxam–Gilbert sequencing lanes specific for purines. The position of the various T_n blocks is indicated at the side of the autoradiograph.

sites containing TpA steps, especially TTAA and TATA. We therefore performed similar experiments with a fragment containing four different arrangements of 6 AT base pairs (A_3T_3, T_3A_3, $(TA)_3$, and TAATTA) together with AATT for comparison. The results of hydroxyl radical footprinting experiments with **5** on this fragment are shown in Figure 2D. Compounds **4**, **5**, and **7** produced DNase I footprints at each of the $(A/T)_6$ sites, while cleavage at AATT was not affected. The footprints at these various sites appeared at different ligand concentrations: cleavage at T_3A_3 and TAATTA was abolished at 0.2 μM **7**, while $(TA)_3$ required higher ligand concentrations (1 μM) to produce a footprint. No data could be obtained for A_3T_3 since this region showed no cleavage in the drug-free controls. The rank order of binding sites for these ligands is T_3A_3 > TAATTA > $(TA)_3$. It therefore appears that the binding strength decreases with increasing numbers of TpA steps. Compound **6** only affected enzyme cleavage at concentrations of 3 μM and above, though once again protection of $(TA)_3$ was weaker. A differential cleavage plot for hydroxyl radical cleavage of this fragment in the presence of 1 μM **5** is shown in Figure 2D. This confirms the binding sites and reveals that between 5–7 residues are protected at each position. Figure 2E shows a similar differential cleavage plot for hydroxyl radical cleavage of the fragment containing blocks of 10 AT base pairs in the presence of 1 μM **5**. These results are in contrast to those with distamycin,[13] which produces two distinct footprints at each AT site. This difference is consistent with the larger binding site size for these ligands, so that only one molecule is bound in each AT tract. Once again, between 5–7 bases are protected at each $(A/T)_{10}$ site, with much weaker binding at AATT. DNase I cleavage of this fragment shows protection at all the $(A/T)_{10}$ sites but reveals that the ligands do not discriminate between the different arrangements of AT base pairs.

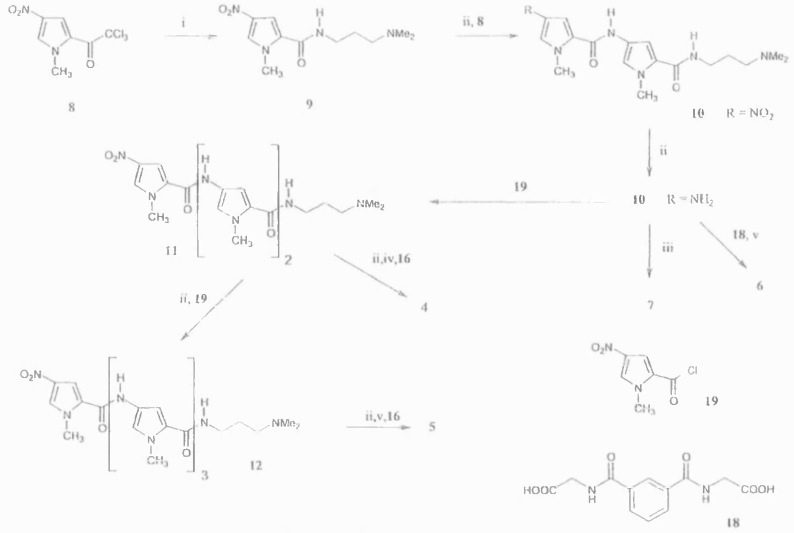
Analogues **4** and **5** both have central regions which are simply extended polypyrroles. Above three pyrrole units, the hydrogen-bonding ability of the amide links ceases to be in-phase with the spacing on the floor of the minor groove,[6,14] resulting in a limit to the reading ability. The good affinity of these two analogues is therefore a reflection of the extra capacity for hydrophobic bonding in the minor groove. Their greater length, compared to the natural ligands, accounts for their preference for extended A/T base pair sequences. The glycine linkers in **6** are spaced appropriately for restoration of phasing with the H-bonding opportunities in the minor groove and were designed to be tolerant of, and perhaps selective toward, GC base pairs.[15] In this case, however, the hydrophilic nature of the amide links may affect binding to DNA and also would prejudice the chances of such a molecule reaching the site of action in vivo. Compound **6** is capable of weakly protecting short AT runs (not shown in the figures) which may indicate that the molecule is only binding at one end, with the central amide groups extending out into water, or that the molecule is behaving as a 'hairpin' and folding back to give side-by-side filling of the minor groove. To do this, at least one of the charges on the end amino groups would have to be lost by deprotonation.

Analogue **7** is a dimethylamino version of a head-to-head-linked netropsin analogue previously reported to display bidentate binding in $(AT)_n$ rich sequences.[16] This relatively rigid linker has a length of 4.2 Å, close to the 4.4 Å required to maintain the ideal phasing of ligand amide hydrogens. Although the central fumaramide unit has the right length, modeling indicates a distortion in the helicity of the molecule in the center, possibly accounting for the slightly weaker binding observed with this analogue.

Chemistry. For the construction of polypyrroles with a dimethylaminoalkyl end group, we used stepwise addition of *N*-methyl-4-nitro-2-trichloroacetylpyrrole units to amino-substituted mono- or polypyrroles with dimethylaminopropylamino tails (Scheme 1). The amino groups were generated by catalytic hydrogenation of the nitro group of the growing chain, an approach which was used to generate oligomers up to four rings in length. Addition of further rings resulted in a rapid decline in yield.

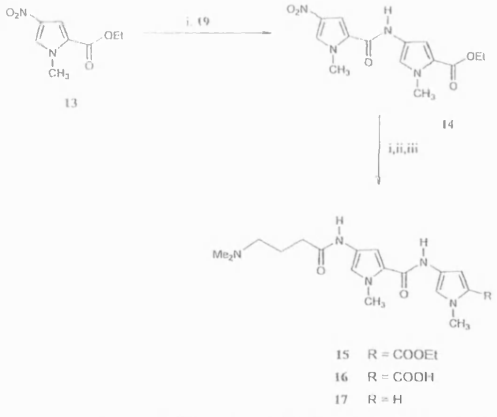
The syntheses of **4** and **5** were therefore based on the coupling of a two-ring and a three- or four-ring unit in a convergent fashion, in good yield (Scheme 1). This route was designed to allow a flexible approach to the synthesis of longer and more complex lexitropsins in the future. Analogue **4** was constructed by coupling a three-ring amine (Scheme 1) to a two-ring acid (Scheme 2), using HBTU/Et_3N, followed by HPLC purification. The hexapyrrole **5** followed similarly, from a four-ring amine and a two-ring acid.

Scheme 1[a]



[a] Reagents: (i) $NH_2(CH_2)_3NMe_2$, rt; (ii) Pd/C/H_2; (iii) fumaric acid, HBTU, NMM; (iv) HBTU, Et_3N, DMF; (v) HBTU, NMM, DMF.

Scheme 2[a]



[a] Reagents: (i) Pd/C/H_2; (ii) $HOOC(CH_2)_3NMe_2$/HBTU/Et_3N/DMF; (iii) NaOH.

The isophthalic diamide **6** was synthesized in good yield, by first treating isophthaloyl chloride with glycine methyl ester to give **18**, followed by hydrolysis and coupling to **10** (R = NH_2), using HBTU/NMM (Scheme 1). Derivative **7** came directly from the coupling of the two-ring amine **10** (R = NH_2), used in the previous syntheses, with fumaric acid (Scheme 1).

Partitioning. Application of eq 1 to distamycin (**2**), which has expected pK_a ca. 12.4 and calculated[17] log P value of −0.87, gives the curve shown in Figure 4, showing the effect of the very strongly basic amidine moiety, cf. acetamidine.[18] At pH 7.4, distamycin is expected to favor the aqueous phase, with potential difficulty in passing through a series of lipid membrane barriers. Calculated values of log P, assumed values of pK_a, and the resulting calculated values of log D at pH 7.4 for compounds **1**−**7** are given in Table 1.

Table 1. Input Data and Calculated log Distribution Coefficients for Extended Lexitropsins

compd	log P[a]	pK_a[b]	log D (pH 7.4)[c]
1	−3.29	12.4	−13.29
2	−0.87	12.4	−5.87
3	2.17	9.5	0.07
4	3.89	9.5	−0.32
5	4.68	9.5	0.47
6	1.47	9.5	−2.74
7	3.18	9.5	−1.03

[a] Calculated using the program in ref 17. [b] Assumed figures. [c] Calculated from eqs 1 and 2.

If the amidine is replaced with a dimethylaminoalkyl substituent, giving **3**, which has been found to be consistent with maintenance of DNA binding,[19] the pK_a is reduced by about 2 log units, by analogy with a range of compounds such as imipramine[20] and log P is increased by ca. 3 log units, giving the curve in Figure 4. Inspection of the curve shows that the behavior in the critical region about pH 7.4 is markedly affected: no longer is there such a strong preference for the aqueous phase. Such an analogue would be expected to show much improved bioavailability.

Relatively small molecules such as **1**−**3** would be expected to be sufficiently water-soluble to be capable of formulation as drugs. Longer DNA reading frames, however, require long stretches of hydrophobic bases, which by definition are likely to be less-water-soluble. Introduction of a second dimethylaminoalkyl group at the opposite end of the molecule to the first one, as in **4**−**7**, offers the possibility of improvement in water solubility.

The two basic centers are sufficiently far apart to behave as individual units, with the same or very

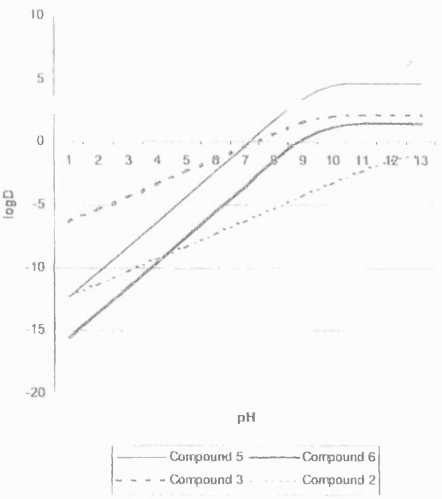


Figure 4. pH-partition behavior of compounds **2**, **3**, **5**, and **6** calculated from eqs 1 and 2.

similar pK_a values, which by analogy[20] we expect to be in the range 9–10. Figure 4 shows the pH-partition behavior of the most (**5**) and least (**6**) lipophilic of the dibasic analogues described in the present study, calculated from eq 2. Comparison of the data in Figure 4 shows the very marked effect of the second basic center, in **5** and **6**, in the region about pH 7.4. It is apparent that such analogues can tolerate much larger lipophilic 'loads' in the central region; of these analogues, the polypyrrole **5**, with the highest calculated value of log *P*, promises the kind of partitioning behavior consistent with good bioavailability. All of the dibasic compounds in the present study were readily water-soluble.

Overall, the significance of these results lies not only with the sequence-reading ability of these particular molecules but in the finding that water solubility can be a designed attribute, without compromising the physicochemical characteristics theoretically required for tissue penetration and maintaining affinity for DNA. It is becoming increasingly apparent, especially with the larger molecules required for extended sequence recognition in DNA, that drug design must take account of solubility and tissue penetration as primary features,[19] alongside receptor affinity and selectivity. In this context, the insertion of extra amide links to change hydrogen-bond phasing, as in **6**, must be approached with caution, since an amide makes a strong negative contribution to the log *P*. However, if attention is paid to the pH-partitioning characteristics of designed molecules, there is no reason lexitropsins cannot be designed to read long lengths of DNA and also have adequate bioavailability.

Experimental Section

Chemistry. ^{1}H NMR spectra were recorded at 400 MHz on a Bruker AMX spectrometer. Electrospray mass spectra were recorded on a Fisons VG Platform Benchtop LC–MS and EIMS on a JEOL JMS-AX505HA mass spectrometer. HPLC purifications were effected on a Vydac Protein and Peptide C18 column using a solvent gradient with two solvents: 0.1% TFA in water (A) and acetonitrile 90%, water 10%, TFA 0.1% (B). Elution commenced with 95% A, 5% B and finished with 100% B. Accurate mass measurements of compounds **4**–**7** were carried out at the EPSRC National Mass Spectrometry Service Centre, University of Wales, Swansea, U.K.

3-(1-Methyl-4-nitropyrrole-2-carboxamido)dimethylaminopropane, 9. A literature procedure[21] gave **9** in 90% yield: mp 130–131 °C (lit.[21] mp 129–130 °C).

3-[1-Methyl-4-(1-methyl-4-nitropyrrole-2-carboxamido)-pyrrole-2-carboxamido]dimethylaminopropane, 10. A literature procedure[21] gave **10** in 78% yield: mp 191–192 °C (lit.[21] mp 190–191 °C).

3-{1-Methyl-4-[1-methyl-4-(1-methyl-4-nitropyrrole-2-carboxamido)pyrrole-2-carboxamido]pyrrole-2-carboxamido}dimethylaminopropane, 11. A literature procedure[21] gave **11** in 63% yield: mp 198 °C (lit.[21] mp 136–137 °C; lit.[22,23] mp 205–206 °C).

(2*E*)-*N*^{1},*N*^{1}-Bis[5-({[5-({[3-dimethylaminopropyl]amino}-carbonyl)-1-methyl-1*H*-pyrrol-3-yl]amino}carbonyl)-1-methyl-1*H*-pyrrol-3-yl]-2-butenediamide Bistrifluoroacetate, 7. 10, R = NO_2 (200 mg, 0.53 mmol), and Pd/C 10% (200 mg) were suspended in 2-propanol (25 mL) and hydrogenated for 4 h at room temperature. Filtration of the catalyst over Kieselguhr under N_2 followed by removal of the solvent in vacuo gave the amine **10**, R = NH_2, as an off white solid that was used without further purification. Fumaric acid (25 mg, 0.22 mmol), HBTU (190 mg) and *N*-methylmorpholine (70 mL) were dissolved in DMF (1 mL, dry), with stirring at room temperature and then left for 30 min. The amine was dissolved in DMF (1.5 mL, dry) and added to the reaction mixture at room temperature with stirring. The reaction mixture was left stirring at room-temperature overnight, then purified by HPLC to give **7** as the bis-TFA salt (75 mg, 35% yield): ^{1}H NMR δ (DMSO-d_6) 1.24–1.84 (4H, p, $2CH_2$), 2.79 (3H, s, NMe), 2.80 (3H, s, NMe), 3.05–3.10 (4H, m, $2CH_2$), 3.24–3.27 (4H, m, $2CH_2$), 3.82 (6H, s, 2NMe, pyrrole), 3.68 (6H, s, 2NMe, pyrrole), 6.93 and 6.94 (2H, d), 6.94 and 6.95 (2H, d), 7.09 (2H, s), 7.16 and 7.17 (2H, d), 8.13–8.16 (2H, t, 2CONH exchangeable), 9.32 (2H, broad 2TFA exchangeable), 9.92 (2H, s, 2CONH exchangeable), 10.47 (2H, s, 2CONH exchangeable); ES-MS found (M + 1) 773.4216, calcd for $C_{38}H_{53}N_{12}O_6$ 773.4211.

3-[1-Methyl-4-(1-methyl-4-(1-methyl-4-{1-methyl-4-nitropyrrole-2-carboxamido}pyrrole-2-carboxamido)pyrrole-2-carboxamido)pyrrole-2-carboxamido]dimethylaminopropane, 12. A mixture of **11** (1.00 g, 2.01 mmol) and Pd/C-10% (1.00 g) in ethanol (100 mL) was hydrogenated at room temperature for 20 h. The catalyst was removed by filtration through Kieselguhr and the filtrate evaporated under reduced pressure to give the crude amine as a pale yellow solid residue (772 mg, 82%). This amine (722 mg, 1.6502 mmol) was dissolved in dry DMF (3.0 mL) and the solution cooled in an ice bath and stirred while a solution of **8** (447 mg, 1.65 mmol) in dry DMF (1.0 mL) was added slowly during a period of 5 min. The resultant mixture was allowed to come to room temperature and stirred for 21 h. Water (30 mL) was added and the oily precipitate was scratched until it solidified. The solid was washed with water and, after drying in vacuo, amounted to 745 mg. Crystallization from aqueous methanol gave **12** (425 mg, 34% yield) as a yellow microcrystalline solid: mp 192–195 °C (lit.[24] mp 195 °C); ES-MS [M + 1] found 622.6, calcd for $C_{29}H_{36}N_{10}O_6$ 621.7; ^{1}H NMR (DMSO-d_6) δ 1.61 (2H, m, CH_2–*CH_2*–CH_2), 2.13 (6H, s, $N(CH_3)_2$), 2.24 (2H, t, CH_2–*CH_2*–NMe_2), 3.21 (2H, m, CONH–*CH_2*–CH_2), 3.80 (3H, s, NMe), 3.85 (3H, s, NMe), 3.87 (3H, s, NMe), 3.97 (3H, s, NMe), 6.83 (1H, d, ArH), 7.06 (2H, d, 2ArH), 7.19 (1H, d, ArH), 7.26 (1H, d, ArH), 7.29 (1H, d, ArH), 7.60 (1H, d, ArH), 8.09 (1H, t, CO-*NH*-CH_2, exchangeable), 8.20 (1H, d, ArH), 9.91 (1H, s, *CONH*, exchangeable), 10.01 (1H, s, *CONH*, exchangeable), 10.31 (1H, s, *CONH*, exchangeable).

Ethyl 1-Methyl-4-(1-methyl-4-nitropyrrole-2-carboxamido)pyrrole-2-carboxylate, 14. The nitro compound **13** (2.057 g, 10.39 mmol) was hydrogenated over 10% Pd/C (805 mg) in ethanol (200 mL) at room temperature for 2 h. The catalyst was removed by filtration through Kieselguhr and the

filtrate evaporated under reduced pressure to leave the crude amine as a gray oil. This oil was dissolved in an ice–water bath, while a solution of **19** (10.39 mmol, prepared[24] from 1.766 g of the carboxylic acid, using 2.0 mL of thionyl chloride, and 8 mL of DME) in dry DME (10 mL) was added slowly over a period of 5 min. The resultant mixture was stirred and allowed to come to room temperature overnight then it was evaporated under reduced pressure. The residue was stirred for 1 h with N HCl (50 mL) and the solid filtered, washed well with ethanol and dried in vacuo to give **14** as a yellow powder (1.377 g, 41% yield): mp 232–234 °C (lit.[25] mp 239–240 °C); IR (KBr) ν_{max} 3358, 3131, 1692, 1663, 1572, 1437, 1391, 1317, 1253, 1121 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.24–1.30 (3H, t, CH_2CH_3), 3.84 (3H, s, NMe), 3.94 (3H, s, NMe), 4.16–4.24 (2H, q, CH_2CH_3), 6.90–6.91 (1H, d), 7.42–7.43 (1H, d), 7.54–7.55 (1H, d), 8.18–8.19 (1H, d), 10.26 (1H, s, *CONH* exchangeable).

Ethyl 1-Methyl-4-(1-methyl-4-(4-dimethylaminobutyryl)-amino)pyrrole-2-carboxylate, 15. A mixture of **14** (605 mg) and Pd/C–10% (627 mg) in ethanol (60 mL) was hydrogenated at room temperature for 16 h. The catalyst was removed by filtration through Kieselguhr and the filtrate was evaporated under reduced pressure to leave the crude amine as a buff colored glassy foam (456 mg, 83% yield) which was used without further purification. A mixture of 4-dimethylamino-butyric acid hydrochloride (527 mg, 3.15 mmol, 2 equiv), HBTU (1.1923 g, 3.15 mmol, 2 equiv), Et_3N (1.31 mL, 6 equiv) and dry DMF (7.0 mL) was placed under N_2 and stirred at room temperature for 30 min. Then to the stirred mixture was slowly added a solution of the above amine (456 mg, 1.57 mmol, 1 equiv) in dry DMF (2.5 mL) over a period of 5 min. After stirring at room temperature for 3 h the resultant mixture was diluted with EtOAc (250 mL) then extracted with 10% Na_2CO_3 (100 mL). The organic layer was washed with brine, dried ($MgSO_4$), and evaporated under reduced pressure to leave a red-brown oil (1.036 g). The crude product was purified by flash column chromatography over silica, using MeOH containing 3% of concentrated ammonia as eluant, to give **15** as an amber oil (487 mg, 77% yield): ES-MS [M + 1] found 404.2, calcd for $C_{20}H_{29}N_5O_4$ 404.5; ^1H NMR (DMSO-d_6) δ 1.24 (3H, t, O–CH_2–CH_3), 1.66 (2H, m, CH_2–CH_2–CH_2), 2.08 (6H, s, NMe_2), 2.20 (4H, m, CH_2–CH_2–CH_2), 3.77 (3H, s, pyrrole NMe), 3.79 (3H, s, pyrrole NMe), 4.17 (2H, q, O–CH_2–CH_3), 6.80 (1H, d, ArH), 7.12 (1H, d, ArH), 7.36 (1H, d, ArH), 9.80 (1H, s, CONH, exchangeable), 9.90 (1H, s, CONH, exchangeable).

1-Methyl-4-(1-methyl-4-(4-dimethylaminobutyryl)amino)pyrrole-2-carboxamido)pyrrole-2-carboxylic Acid, 16. A solution of **15** (465 mg, 1.15 mmol) in EtOH (5 mL) and NaOH (1.73 mmol) was heated under reflux for 1 h and then concentrated under reduced pressure. The residue was dissolved in MeOH (5 mL) containing 3% of concentrated NH_4OH, and the resultant solution was applied to a flash column of silica. The column was eluted first with a mixture of EtOAc (60 parts) and MeOH containing 3% concentrated NH_4OH (40 parts), to elute any unreacted ester **15** and any decarboxylation product **17**, then MeOH containing 3% of concentrated NH_4OH was used to elute the carboxylic acid **16**. After evaporation water was removed from the residue by coevaporation with EtOH, and finally the residue was triturated with Et_2O until it solidified. After drying in vacuo the product was obtained as a cream-colored powder (258 mg, 60% yield): mp 138 °C (gas evolution); ES-MS [M + 1] found 376.2, calcd for $C_{18}H_{25}N_5O_4$ 376.4; IR (KBr) ν_{max} 1639, 1617, 1566 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.69 (2H, m, CH_2–CH_2–CH_2), 2.14 (6H, s, NMe_2), 2.26 (4H, m, CH_2–CH_2–CH_2, after the addition of D_2O this became 2H, t, and a new triplet for 2H appeared at 2.85), 3.81 (6H, s, 2 × pyrrole NMe), 6.78 (1H, d, ArH), 6.85 (1H, d, ArH), 7.15 (1H, d, ArH), 7.37 (1H, d, ArH), 9.82 (1H, s, exchangeable, CONH), 9.83 (1H, s, exchangeable, CONH). The CO_2H signal was not observed.

Note: It was inadvisable to acidify the product of this hydrolysis. Whenever this was done, either by using HCl, or by attempting to isolate the product by reverse-phase HPLC using an acidic (TFA) solvent system, considerable decarboxylation occurred to give **17**. This decarboxylation product had a very close HPLC retention time to that of **16**, but separation could be accomplished using flash column chromatography over silica using EtOAc/MeOH/NH_4OH as eluant as described above. Compound **17** was thus isolated from one experiment as an amber gum: ES-MS 332.1, calcd for $C_{17}H_{25}N_5O_2$ 332.4 [M + 1]; ^1H NMR (DMSO-d_6) δ 1.79 (2H, m, CH_2–CH_2–CH_2), 2.28 (2H, m, CH_2), 2.34 (6H, s, NMe_2), 3.38 (2H, m, CH_2), 3.55 (3H, s, pyrrole NMe) 3.80 (3H, s, pyrrole NMe), 6.05 (1H, m, ArH), 6.52 (1H, m, ArH), 6.81 (1H, d, ArH), 7.08 (1H, m, ArH), 7.13 (1H, d, ArH), 9.71 (1H, s, exchangeable, CONH), 9.86 (1H, s, exchangeable, CONH).

3-(1-Methyl-4-(1-methyl-4-(1-methyl-4-(1-methyl-4-(1-methyl-4-(4-dimethylaminobutyryl)aminopyrrole-2-carboxamido)pyrrole-2-carboxamido)pyrrole-2-carboxamido)pyrrole-2-carboxamido)pyrrole-2-carboxamido)dimethylaminopropane Bistrifluoracetate, 4. A mixture of **11** (151 mg) and Pd/C–10% (164 mg) in EtOH (15 mL) was hydrogenated at room temperature for 13 h. The catalyst was removed by filtration through Kieselguhr, and the filtrate evaporated under reduced pressure to leave the crude amine (128.5 mg, 90% yield) as a pale yellow solid. This material was used at once without further purification. A mixture of the carboxylic acid **16** (68.6 mg, 0.18 mmol, 1 equiv), HBTU (104.1 mg, 0.275 mmol, 1.5 equiv), Et_3N (76 mL, 55.5 mg, 0.55 mmol, 3 equiv) and dry DMF (1.5 mL) was placed under N_2 and stirred at room temperature for 30 min. To the resultant clear solution was added a solution of the above crude amine (128.5 mg, 0.275 mmol, 1.5 equiv) in dry DMF (1.0 mL), and the mixture stirred at room temperature for 8 h. The resultant suspension was purified by reverse-phase HPLC. Fractions containing the product were frozen immediately after collection and then freeze dried to give the bis-TFA salt **4** as a fawn powder (65.3 mg, 34% yield): ES-MS found [M + 1] 826.4476, calcd for $C_{41}H_{56}N_{13}O_6$ 826.4476; ^1H NMR (DMSO-d_6) δ 1.82–1.93 (4H, 2 × overlapping quintets, 2 × CH_2–CH_2–CH_2), 2.35 (2H, t, CH_2–CH_2–CO), 2.79 (12H, m, 2 × NMe_2), 3.07 (4H, m, 2 × CH_2–CH_2–NMe_2), 3.24 (2H, m, NH–CH_2–CH_2), 3.72–3.86 (15H, 5 × pyrrole NMe), 6.87–7.22 (10H, 10 × ArH), 8.14 (1H, t, exchangeable *CONH*–CH_2), 9.2–9.5 (2H, broad, exchangeable, 2 × TFA), 9.89–9.94 (5H, exchangeable, 5 × CONH).

3-(1-Methyl-4-(1-methyl-4-(1-methyl-4-(1-methyl-4-(1-methyl-4-(1-methyl-4-(4-dimethylaminobutyryl)amino-pyrrole-2-carboxamido)pyrrole-2-carboxamido)pyrrole-2-carboxamido)pyrrole-2-carboxamido)pyrrole-2-carboxamido)dimethylaminopropane Bistrifluoracetate, 5. The nitro compound **12** (1125 mg) was hydrogenated in a mixture of MeOH (10.5 mL) and 0.1 N HCl (4.5 mL) over Pd/C–10% (125 mg) as described in the previous experiment above, to afford, after freeze-drying, the crude amine (112.5 mg, 84%). This amine was mixed with the carboxylic acid **16** (42.4 mg, 0.11 mmol), HBTU (64.2 mg, 0.17 mmol), NMM (50 mL) and DMF(1.0 mL, dry), and the resultant mixture was stirred at room temperature for 24 h. The product was isolated by means of reverse-phase HPLC. Fractions containing the product were frozen immediately on collection then freeze-dried to give the bis-TFA salt of **5** as a buff-colored powder (58.3 mg, 37% yield) which had no distinct melting point: ES-MS found [M + 1] 948.4964, calcd for $C_{47}H_{62}N_{15}O_7$ 948.4956; ^1H NMR (DMSO-d_6) δ 1.82–1.93 (4H, 2 × overlapping quintets, 2 × CH_2–CH_2–CH_2), 2.35 (2H, t, CH_2–CH_2–CO), 2.79 (12H, m, 2 × NMe_2), 3.07 (4H, m, 2 × CH_2–CH_2–NMe_2), 3.24 (2H, m, NH–CH_2–CH_2), 3.82–3.87 (18H, 6 × pyrrole NMe), 6.87–7.22 (12H, 12 × ArH), 8.15 (1H, t, exchangeable, *CONH*–CH_2), 9.30 (1H, broad, exchangeable, TFA), 9.50 (1H, broad, exchangeable, TFA), 9.90–9.94 (6H, exchangeable, 6 × *CONH*).

Isophthalamidodiacetic Acid, 18. A stirred suspension of isophthaloyl chloride (200 mg, 1.00 mmol) and glycine methyl ester hydrochloride (275 mg, 2.20 mmol) in anhydrous methylene chloride (20 mL) was treated dropwise with a solution of triethylamine (0.66 g, 6.60 mmol) in dry methylene chloride (5 mL). The resulting solution was then stirred at

room temperature for 1.5 h. The solvent was evaporated and the residue treated with 2 M HCl (5 mL) then extracted with methylene chloride. Evaporation of the extract gave the required product as a pale yellow gum (210 mg, 75% yield): ^{1}H NMR (DMSO-d_6) δ 3.66 (6H, s, 2 × OMe), 4.04 (4H, d, *J* 6 Hz, 2 × NHCH_2, collapses to a singlet on addition of D_2O), 7.62 (1H, t, *J* 8 Hz, ArH), 8.03 (2H, dd, J_o 8 Hz, J_m 2 Hz, 2 × ArH), 8.38 (1H, m, ArH), 9.12 (2H, t, *J* 6 Hz, *COHN*); EI-MS found 308.1026, calcd for $C_{14}H_{16}N_2O_6$ 308.2220. A solution of the dimethyl ester (690 mg, 2.30 mmol) in ethanol (30 mL) was treated with 2 M NaOH (15 mL) and the resulting solution stirred at room temperature for 1 h. The mixture was concentrated by evaporation to a total volume of 10–15 mL, then acidified with 2 M HCl and extracted with ethyl acetate. Evaporation of the extract gave **18** as a colorless solid (260 mg, 41% yield): mp 152–154 °C; ^{1}H NMR (DMSO-d_6) δ 3.94 (4H, d, *J* 6 Hz, CH_2 collapses to a singlet on addition of D_2O), 7.62 (1H, d, *J* 8 Hz, ArH), 8.03 (2H, dd, J_o 8 Hz, J_m 2 Hz, 2 × ArH), 8.37 (1H, s, ArH), 8.98 (2H, t, *J* 6 Hz, 2 × *CONH*CH_2 exchangeable), 13.12 (2H, broad, CO_2H exchangeable); ES-MS found 279.01 [M − 1], calcd for $C_{12}H_{12}N_2O_6$ [M − 1] 279.17.

N^1,N^9-Bis(2-{[5-({[5-({[3-dimethylaminopropyl]amino}carbonyl)-1-methyl-1*H*-pyrrol-3-yl]amino}carbonyl)-1-methyl-1*H*-pyrrol-3-yl]amino}-2-oxoethyl)isophthalamide Bistrifluoracetate, 6. A solution of the dicarboxylic acid **18** (28 mg, 0.1 mmol), NMM (30 mg, 0.3 mmol) and HBTU (165 mg, 0.3 mmol) in DMF (1 mL, dry) was stirred at room temperature for 1 h. The mixture was then treated with a solution of freshly prepared **10**, R = NH_2 [prepared beforehand by hydrogenating a suspension of **10**, R = NO_2 (165 mg, 0.3 mmol) with Pd/C–10% (165 mg) in 2-propanol (20 mL) for 6 h] in dry DMF (1 mL). The resulting solution was stirred at room temperature overnight. The mixture was then purified by HPLC to give the required product (60 mg, 52% yield) as a pale yellow solid (bis-TFA salt) with no distinct melting point: ^{1}H NMR (DMSO-d_6) δ 1.83 (4H, m, *J* 7 Hz, $CH_2CH_2CH_2$), 2.78 (12H, d, *J* 4 Hz, 4 × NHMe_2), 3.07 (4H, m, CONHCH_2, collapses to a triplet on addition of D_2O), 3.24 (4H, m, 2 × Me_2NHCH_2, collapses to a triplet on addition of D_2O), 3.81 (6H, s, 2 × NMe), 3.83 (6H, s, 2 × NMe), 4.04 (4H, d, *J* 6 Hz, NHCH_2CO, collapses to a singlet on addition of D_2O), 6.91–6.93 (4H, m, 2 × ArH), 7.15 (2H, d, *J* 2 Hz, ArH), 7.17 (2H, d, *J* 2 Hz, ArH), 7.61 (1H, t, J_o 8 Hz, ArH), 8.06 (2H, dd, J_o 8 Hz, J_m 2 Hz, ArH), 8.14 (2H, t, *J* 6 Hz, 2 × *NH*CH_2, exchangeable), 8.43 (1H, s, ArH), 8.91 (2H, t, *J* 6 Hz, 2 × *CONH*, exchangeable), 9.27 (2H, broad, 2 × *NH*Me_2, exchangeable), 9.85 (2H, s, 2 × *CONH*, exchangeable), 9.96 (2H, s, 2 × *CONH*, exchangeable); ES-MS found [M + 1] 937.4804, calcd for $C_{46}H_{61}N_{14}O_8$ 937.4797.

Footprinting Studies. Ligands: The ligands were dissolved in 10 mM Tris-HCl pH 7.5 containing 10 mM NaCl at a concentration of 10 mM and stored at −20 °C. The compounds were diluted to working concentrations in the same buffer immediately before use. Oligonucleotides for preparing the DNA fragments containing synthetic DNA inserts were purchased from Oswel. DNase I was purchased from Sigma and stored at −20 °C at a concentration of 7200 units/mL.

DNA fragments: The sequences of the five most significant DNA fragments used in this work are shown in Figure 1. The TyrT fragment, derived[25] from plasmid pKMΔ-98, was obtained by digesting the plasmid with *Eco*R1 and *Ava*1 and labeled at the 3′-end of the *Eco*R1 site with [α-^{32}P]dATP using reverse transcriptase. Plasmid pAAD1 was prepared as previously described.[2] The other plasmids were prepared by cloning oligonucleotides into the *Sma*I site of pUC18 as previously described. pAAD1[2] contains an insert which possesses 5 different $(A/T)_4$ tracts, separated by 6 GC base pairs (CGCGCG). Plasmid pA_{3-6} contains 4 $T_n \times A_n$ tracts of varying length (n = 3–6) separated by 4 GC base pairs (CGCG). $p(A/T)_{6-12}$ contains 4 AT tracts of 6, 8, 10, and 12 bases long with the sequence A_nTAT_n (n = 2–5) separated by 4 GC base pairs (CCGG). Plasmid $p(AT)_6$ contains 4 different arrangements of $(A/T)_6$ sites together with the sequence AATT, each separated by 4 GC base pairs (CGCG). Plasmid $p(A/T)_{10}$ contains 3 different arrangements of $(A/T)_{10}$ sites together with the sequence AATT, each separated by 4 GC base pairs (CGCG). Polylinker DNA fragments containing each of these synthetic sequences were obtained by digesting with *Hin*dIII, labeling with [α-^{32}P]dATP using reverse transcriptase and digesting again with either *Eco*R1 ($p(A/T)_{6-12}$ and pA_{3-6}) or *Sac*I ($p(A/T)_{10}$ and pAAD1). The labeled DNAs of interest were separated from the remainder of the plasmid on 6–8% polyacrylamide gels and dissolved in 10 mM Tris-HCl pH 7.5 containing 0.1 mM EDTA.

DNase I footprinting: Radiolabeled DNA (1.5 μL) was mixed with 1.5 μL of ligand (dissolved in 10 mM Tris-HCl pH 7.5 containing 10 mM NaCl) and left to equilibrate at room temperature for at least 30 min. 2 μL of DNase I (approximately 0.01 units/mL diluted in 20 mM NaCl containing 2 mM $MgCl_2$ and 2 mM $MnCl_2$) was then added. The reaction was stopped after 1 min by adding 4 μL of formamide containing 10 mM EDTA and 0.1% (w/v) bromophenol blue.

Hydroxyl radical footprinting: Radiolabeled DNA (2 μL) was mixed with 2 μL of ligand (dissolved in 10 mM Tris-HCl pH 7.5 containing 10 mM NaCl) and left to equilibrate at room temperature for at least 30 min. Digestion was initiated by adding 6 μL of a freshly prepared solution containing 30 μM ferrous ammonium sulfate, 300 μM EDTA, 3 mM ascorbic acid and 0.1% H_2O_2. The reaction was stopped after 10 min by adding 8 μL of 0.75 M sodium acetate and 60 μL of ethanol. The DNA was precipitated, washed with 70% ethanol, dried and redissolved in 8 mL of formamide containing 10 mM EDTA and 0.1% (w/v) bromophenol blue.

Gel electrophoresis: Samples were denatured by boiling for 3 min, cooled on ice and loaded onto denaturing polyacrylamide gels (6–10% w/v) containing 8 M urea. Gels (40 cm long) were run at 1500 V for about 2 h before fixing in 10% acetic acid (v/v), transferring to Whatman 3MM paper, drying under vacuum at 80 °C and exposing to autoradiography at −70 °C with an intensifying screen. Autoradiographs were scanned with a Hoefer 365W scanning microdensitometer. The intensity of each band was estimated using the Hoefer software. Differential cleavage plots, calculated from these scans, represent the intensity of each band in the drug-treated lane divided by the intensity of the corresponding band in the drug-free control.

References

(1) Wartel, R. M.; Larson, R. M.; Wells, R. D. A specific probe for A–T regions of duplex deoxyribonucleic acid. *J. Biol. Chem.* **1974**, *249*, 6719–6731.

(2) Abu-Daya, A.; Brown, P. M.; Fox, K. R. DNA sequence preferences of several AT-selective minor groove binding ligands. *Nucleic Acids Res.* **1995**, *23*, 3385–3392.

(3) Hamdan, I. I.; Skellern, G. G.; Waigh, R. D. Ligand binding to oligonucleotides. *Methods Mol Biol.*, in press.

(4) For example: Rao, K. E.; Shea, R. G.; Bathini, Y.; Lown, J. W. Molecular recognition between oligopeptides and nucleic acids – DNA sequence specificity and binding properties of thiazole-lexitropsins incorporating the concepts of base site acceptance and avoidance. *Anti-Cancer Drug Des.* **1990**, *5*, 3–20. Dwyer, T. J.; Geierstanger, B. H.; Bathini, Y.; Lown, J. W.; Wemmer, D. E. Design and binding of a distamycin-A analogue to d(CGCAAGTTGGC)·d(GCCAACTTGCG) – synthesis, NMR studies and implications for the design of sequence-specific minor groove binding oligopeptides. *J. Am. Chem. Soc.* **1992**, *114*, 5912–5919. Geierstanger, B. H.; Mrksich, M.; Dervan, P. B.; Wemmer, D. E. Design of a C·C specific DNA minor-groove binder. *Science* **1994**, *266*, 646–650.

(5) Turner, J. M.; Baird, E. E.; Dervan, P. B. Recognition of seven base pair sequences in the minor groove of DNA by ten-ring pyrrole-imidazole polyamide hairpins. *J. Am. Chem. Soc.* **1997**, *119*, 7636–7644.

(6) For example: Guo, D.; Gupta, R.; Lown, J. W. DNA sequence-selective binding of head-to-tail linked bis-lexitropsins – relation of phasing to cytotoxic potency. *Anti-Cancer Drug Des.* **1993**, *8*, 369–397.

(7) Trauger, J. W.; Baird, E. E.; Dervan, P. B. Recognition of DNA by designed ligands at subnanomolar concentrations. *Nature* **1996**, *382*, 559–561. Herman, D. M.; Baird E. E.; Dervan, P. B. Stereochemical control of the DNA binding affinity, sequence specificity and orientation preference of chiral hairpin polyamides in the minor groove. *J. Am. Chem. Soc.* **1998**, *120*, 1382–1391.

(8) Haq, I.; Ladbury, J. E.; Chowdrhy, B. Z.; Jenkins, T. C.; Chaires, J. B. Specific binding of Hoechst 33258 to the (CGCAAATT·TGCG)$_2$ duplex: calorimetric and spectroscopic studies. *J. Mol. Biol.* **1997**, *271*, 244−257.
(9) For example: Singh, M. P.; Plouvier, B.; Hill, G. C.; Gueck, J.; Pon, R. T.; Lown, J. W. Isohelicity and strand selectivity in the minor groove binding of chiral (1R,2R)-bis(netropsin)-1,2-cyclopropanedicarboxamide and (1S,2S)-bis(netropsin)-1,2-cyclopropanecarboxamide ligands to duplex DNA. *J. Am. Chem. Soc.* **1994**, *116*, 7006−7020. Wang, A. H. J.; Cottens, S.; Dervan, P. B.; Yesinowski, J. P.; Vandermarel, G. A.; Vanboom, J. H. Interactions between a symmetrical minor groove binding compound and DNA oligonucleotides — H-1 and F-19 NMR studies. *J. Biomol. Struct. Dyn.* **1989**, *7*, 101−117.
(10) Florence, A. T.; Attwood, D. *Physicochemical Principles of Pharmacy*, 3rd ed.; Macmillan: London, 1998; p 185.
(11) Fox, K. R.; Waring, M. J. DNA structural variations produced by actinomycin and distamycin as revealed by DNAase 1 footprinting. *Nucleic Acids Res.* **1984**, *12*, 9271−9285.
(12) Portugal, J.; Waring, M. J. Hydroxyl radical footprinting of the sequence-selective binding of netropsin and distamycin to DNA. *FEBS Lett.* **1987**, *225*, 195−200.
(13) Abu-Daya, A.; Fox, K. R. Interaction of minor groove binding ligands with long AT tracts. *Nucleic Acids Res.* **1997**, *25*, 4962−4969 and unpublished observations.
(14) Lown, J. W. Lexitropsins − rational design of DNA-sequence reading agents as novel anti-cancer agents and potential cellular probes. *Anti-Cancer Drug Des.* **1988**, *3*, 25−40.
(15) Waszkowycz, B. Unpublished data.
(16) Rao, K. F.; Zimmerman, J.; Lown, J. W. Sequence-selective DNA-binding by linked bis-*N*-methylpyrrole dipeptides − an analysis by MPE footprinting and force-field calculations. *J. Org. Chem.* **1991**, *56*, 786−797.
(17) Leo, A. The octanol−water partition coefficient of aromatic solutes − the effect of electronic interactions, alkyl chains, hydrogen bonds and ortho-substitution. *J. Chem. Soc., Perkin Trans. 2* **1983**, 825−838 and references cited therein.
(18) Sykes, P. *A Guidebook to Mechanism in Organic Chemistry*, 4th ed.; Longman: London, 1975; p 68.
(19) Zhao, R.; Lown, J. W. Synthesis of a novel N-methoxy-methylpyrrole-containing DNA minor-groove binding oligopeptide related to distamycin. *Heterocycles* **1995**, *41*, 337−344.
(20) Green, A. L. Ionization constants and water solubilities of some aminoalkylphenothiazine tranquillizers and related compounds. *J. Pharm. Pharmacol.* **1967**, *19*, 10−16.
(21) Nishiwaki, E.; Tanaka, S.; Lee, H.; Shibuya, M. Efficient synthesis of oligo-*N*-methylpyrrolecarboxamides and related compounds. *Heterocycles* **1988**, *27*, 1945−1952.
(22) Glibin, E. N.; Tsukerman, B. V.; Ginzburg, O. F. Analogue of distamycin A with a dimethylamino group. *J. Org. Chem. USSR (Engl. Transl.)* **1977**, *13*, 2082−2083.
(23) Glibin, E. N.; Tsukerman, B. V.; Ginzburg, O. F. Synthesis of actinomycin analogues. X. Polyfunctional DNA complexing agents. *J. Org. Chem. USSR (Engl. Transl.)* **1981**, *17*, 568−572.
(24) Arcamone, F.; Nerviano, M.; Mongelli, N.; Penco, S. Poly-4-aminopyrrole-2-carboxamide derivatives, procedures for their preparation, and antiviral and antineoplastic pharmaceuticals containing them. Ger. Offen. DE 3,623,880, Jan 29, 1987.
(25) Drew, H. R.; Travers, A. A. DNA structural variations in the *E. coli tyrT* promoter. *Cell* **1984**, *37*, 491−502.
(26) Turchin, K. F.; Grokhovskii, S. L.; Zhuze, A. L.; Gottikh, B. P. Ligands possessing affinity for definite pairs of DNA bases. II. A study of the stereochemistry of the chromophore of distamycin A by ^{1}H NMR spectroscopy. *Sov. J. Bioorg. Chem. (Engl. Transl.)* **1978**, *4*, 780−790.

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The Synthesis of Some Head to Head Linked DNA Minor Groove Binders

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Abstract—A series of head to head linked dimers of heterocyclic amino acids has been prepared for investigation of affinity and selectivity in binding to the minor groove of DNA. The selection of targets for synthesis was led by computer based design. Several novel dicarboxylic acid linkers including indoles, phenanthrenes, a fluorenone, and a bisbenzothiophene have been included. Analysis of binding to DNA by footprinting showed high affinity for compounds derived from 2,7-dihydrophenanthrene dicarboxylic acid and a predominant selectivity for AT rich regions containing at least 4 AT pairs but with the ability to span up to two CG base pairs. © 2000 Elsevier Science Ltd. All rights reserved.

The importance of nucleic acids as a target for drug action has been widely recognised and the potential benefits of controlling the biochemistry of an organism at the level of protein synthesis compared with enzyme inhibition emphasised.[1] A requirement for the effectiveness of drugs that act at nucleic acids is the ability to recognise defined sequences of DNA so that specific genes or control regions can be targeted. Moreover, high affinity for such sites is essential. Natural products such as distamycin are oligomers of heterocyclic amino acids and have been shown to bind to the minor groove of DNA displacing the hydrogen bonded water.[2] Typically they show a preference for AT rich regions and have dissociation constants of the order of 10^{-5} M.[3] Such compounds have intrinsic biological activity but they also have many limitations including toxicity, and low affinity, specificity, and solubility. Approaches to tackle the problems of affinity and specificity have centred on the introduction of additional monomers such as imidazole derivatives to promote CG reading,[4] on the linking of heterocyclic amino acids head to tail by aliphatic amino acids with flexible chains,[5] and on the linking of oligomers of heterocyclic amino acids head to head.[6] An approach from our laboratories to deal with problems of solubility and transport is described in another paper.[7] In this paper we describe the synthesis of a number of novel head to head linked oligomers of heterocyclic amino acids and demonstrate strong affinity when certain polycyclic aromatic compounds are used as linkers.

Keywords: DNA; minor groove; lexitropsin dimer; synthesis.
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Selection of Compounds for Synthesis

In order to optimise affinity of minor groove binders such as the lexitropsins, it is necessary to maximise the hydrogen bonding between the NH of the carboxamides and the 2-carbonyl group of thymine and the 1N of adenine. The spacing of the NH groups in an extended lexitropsin does not closely match the positions of the hydrogen bond acceptors in DNA. Linking molecules were therefore designed to have the correct length and curvature to ensure that hydrogen bonding was possible using the pyrrole amino acid amides on both sides of the linker. A series of aromatic dicarboxylic acids was assessed for their ability to correct ligand misalignment with the DNA bases by computer simulation with an in vacuo DNA model using AMBER 4.0. Fig. 1 shows the shape of the dihydrophenanthrene linked compound in comparison with distamycin. The DNA phosphate groups were neutralised and the ligand termini were truncated to neutral $CONH_2$ groups. The ligands were docked into the minor groove of the hexadecamer $(d(A)\cdot d(T))_{16}$ in the standard Arnott B-DNA conformation and minimised prior to molecular dynamics at 300 K for 200 ps. Geometries from dynamics trajectories were saved at 5 ps intervals for minimisation and subsequent energetic analysis of ligand internal energies and interaction energies between ligand and DNA. The internal energies of minimised isolated ligands were then subtracted from the ligand

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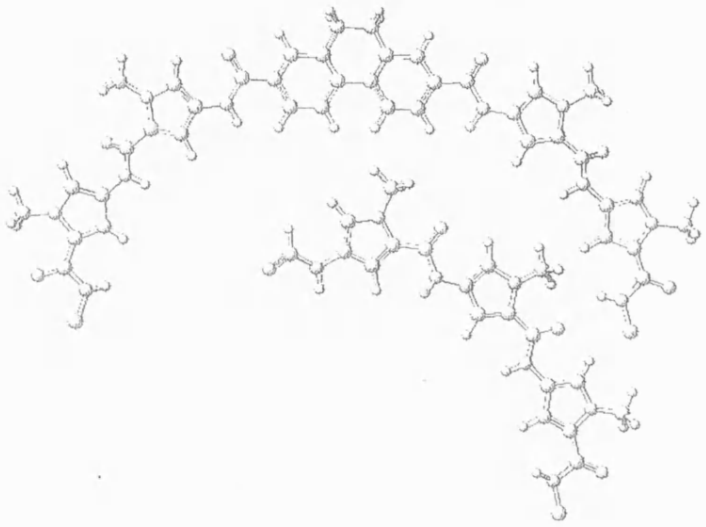


Figure 1. Molecular modelling representation of the cyclic portion of target molecules **19**–**24**, all of which contain a dihydrophenanthrene linker. The tripyrrole of distamycin is shown for comparison. The flexible side chains of both compounds are omitted for clarity. (Figures produced by molecular mechanics energy minimisation using the CACHE molecular modelling software system, Oxford Molecular.)

internal energies from simulation with the DNA model to give a quantitative measure of the ligand distortion upon binding. This distortion term was then added to the interaction energy for each ligand to give a corrected interaction energy, which was then used to rank the linker structures (Table 1). The interactions of cations with anionic sites with DNA was considered to be constant in the evaluation of the linkers themselves. One of the compounds included (**30**) has been synthesised before.[6]

To expand the structural repertoire, a number of compounds containing flexible components were also synthesised (indole derivatives and glycine derivatives: Table 1). For synthetic convenience, tertiary alkyl amines were commonly used as tail groups; this choice is also significant in the context of designing compounds with suitable solubility for use as drugs that will reach targets by passive diffusion.[7,8] Some amidines were also synthesised to provide congruence with previous work.[6] With the rigid linkers, all of these molecules would be expected to bind to DNA predominantly in a 1:1 manner unlike those in which extended flexible linkers such as GABA are included; in such cases, a hairpin or 2:1 structure is formed with an expanded minor groove which facilitates recognition of CG regions through hydrogen bonding to imidazole amino acids.[5]

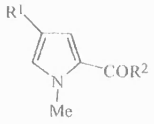
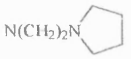
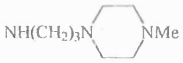
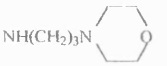
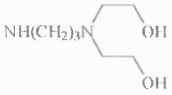
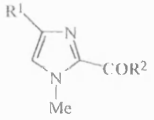
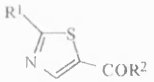
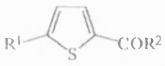
Synthesis of the Heterocyclic Amino Acid Oligomers

The monomeric heterocyclic amino acids (Table 2) were synthesised either directly by ring synthesis (for the thiazole **3**)[9] or by reduction of the corresponding nitro compound (for **1**,**2**,**4**).[10] The terminal carboxyl group was capped using either a monomer or a dimer as substrate with an aminopropyl amine or a cyanopropyl amine. The amidines were prepared by means of the Pinner reaction from the corresponding nitriles.[11] For the capping reactions of the

Table 1. Designed linkers and their calculated (and observed) interaction properties with DNA as shown by footprinting. Compounds marked * were investigated with amidinium headgroups; the remaining compounds had dimethylamino headgroups. Footprinting was carried out as described in Ref. 7. Strong footprint means that protection was observed at a ligand concentration of 0.3 μM; Good footprint refers to a concentration between 1 and 10 μM; '*nt*' means not tested in these experiments

Linker name and target compound number	Net interaction energy (units)	Rank	Observed interaction with DNA
Indole-2,6-dicarboxylate - flexible **33**	−108.9	1	Good footprint
Dihydrophenanthrene dicarboxylate **19**	−106.6	2	Strong footprint*
Bisbenzothiophene dicarboxylate **26**	−105.5	3	Strong footprint*
Phenanthrene dicarboxylic acid **25**	−103.5	4	*nt*
Indole-2,6-dicarboxylate-rigid **32**	−100.9	5	Good footprint
Terephthalic acid **28**	−100.4	6	Good footprint
Fumaric acid **29**	−95.1	7	Good footprint
Indole-2,5-dicarboxylate **35**	−92.6	8	Good footprint*
Indole-2,6-dicarboxylate **31**	−92.1	9	Weak footprint*
Trans-cyclopropane dicarboxylate **30**	−88.6	10	*nt*

Table 2. Monomers synthesised and their abbreviations

Structure	Substituents R^1	Substituents R^2	Abbreviation O_2N pyrr OEt	No.
	NO_2	OEt	O_2N pyrr OEt	**1a**
	NO_2	OH	O_2N pyrr OH	**b**
	NO_2	Cl	O_2N pyrr Cl	**c**
	NO_2	CCl_3	O_2N pyrr CCl_3	**d**
	NO_2	$NH(CH_2)_3NMe_2$	O_2N pyrr dmap	**e**
	NO_2		O_2N pyrr pyrp	**f**
	NO_2		O_2N pyrr mpip	**g**
	NO_2		O_2N pyrr morphp	**h**
	NO_2		O_2N pyrr dheap	**i**
	NO_2	OH	O_2N imid OH	**2a**
	NO_2	$NH(CH_2)_3NMe_2$	O_2N imid dmap	**b**
	NO_2	OEt	O_2N thia OH	**3a**
	NO_2	$NH(CH_2)_3NMe_2$	O_2N thia dmap	**b**
	NO_2	OH	O_2N thio OH	**4a**
	NO_2	$NH(CH_2)_3NMe_2$	O_2N thio dmap	**b**

imidazoles **2a**, pivalic anhydride mediated coupling was the best method found. For the pyrrole and thiophene derivatives, the amides were readily formed under Schotten Baumann conditions from the appropriate acid chloride typically in yields in excess of 85%. Alternatively 4-nitro-*N*-methylpyrrole 2-trichloromethyl ketone was an effective acylating agent for the synthesis of capped monomers.[12] Extension of the monomers to capped oligomers (**5–9**, Table 3) was essentially a two-stage process. Firstly the nitromonomer was hydrogenated using Pd–C as catalyst typically in methanol/ethylene glycol dimethyl ether (DME) mixtures or in DME alone. However not all compounds proved amenable to hydrogenation, the nitrothiophenes requiring extensive research to find appropriate reaction conditions. The amines thus produced were not isolated and purified but used directly for coupling with the second heterocyclic amino acid. The best conditions for coupling closely mirrored those found for the capping of the corresponding monomer. Coupling yields were in the range 58–95%, the best yields being obtained using acid chlorides. On the other hand, acid chlorides were not used in the preparation of the head to head linked compounds (see below).

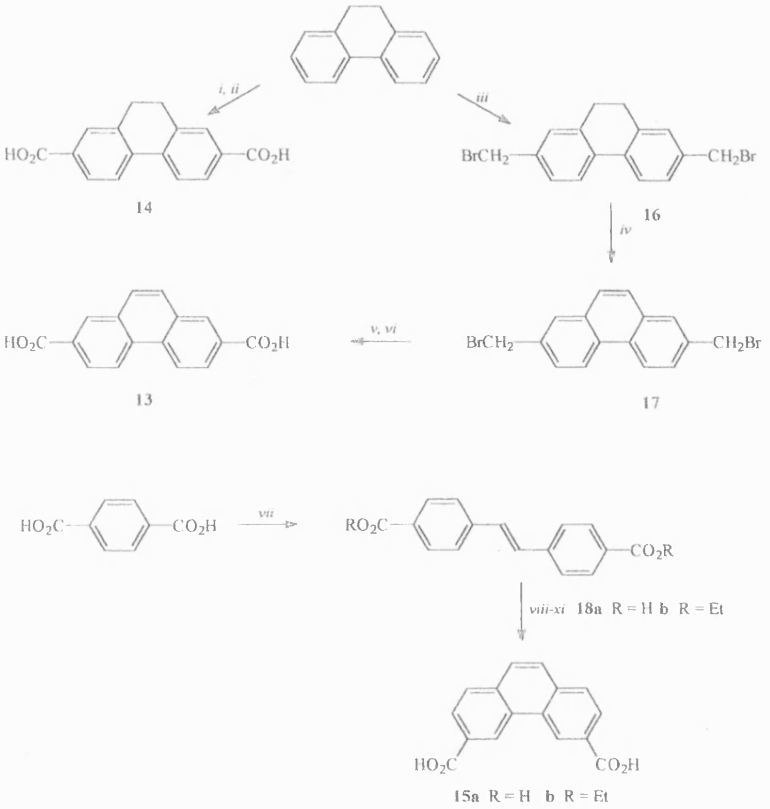
Table 3. Capped oligomers synthesised listed using abbreviations defined by structures in Table 2 above

Compound	No.	Compound	No.
O_2N pyrr pyrr dmap	**5a**	O_2N pyrr pyrr dheap	**5d**
O_2N pyrr pyrr pyrp	**5b**	O_2N pyrr pyrr morphp	**5e**
O_2N pyrr pyrr mpip	**5c**	O_2N pyrr pyrr amidp	**5f**
O_2N imid imid dmap	**6**	O_2N pyrr pyrr cyanp	**5g**
O_2N thio thio dmap	**7**	O_2N pyrr pyrr imidp	**5h**
O_2N pyrr thia dmap	**8**	Zgly pyrr pyrr dmap	**5i**
O_2N pyrr thio dmap	**9**	Gly pyrr pyrr dmap	**5j**

Synthesis of Linkers

The computational design suggested that (a) tricyclic aromatic compounds such as phenanthrene and fluorene derivatives and (b) 2,5- and 2,6-substituted indole dicarboxylic acids would be appropriate components. The synthesis of the phenanthrene derivatives is shown in Scheme 1. Substantial synthetic effort was required to obtain the indole dicarboxylic acids (**10a–e**, Table 4) and that work will be described in another paper.[13] Bisbenzothiophene-2,7-dicarboxylic acid **11** and fluorenone-2,7-dicarboxylic acid **12** were commercial samples.[14]

Three examples already described were included to provide controls (fumarate, *trans*-1,2-cyclopropane dicarboxylate, and terephthalate).[6] For phenanthrene derivatives (**13–15**, Scheme 1), dihydrophenanthrene was bisbromomethylated using paraformaldehyde in the presence of hydrobromic acid[15] to afford **16** which was dehydrogenated using



Scheme 1. *Reagents:* (i) CH_3COCl, $AlCl_3$; (ii) I_2, pyridine then NaOH/aq. EtOH; (iii) $(CH_2O)_n$, HBr, H_3PO_4 in aq HOAc; (iv) DDQ, benzene; (v) DMSO, $NaHCO_3$; (vi) $bipyH_2CrOCl_5$, CH_2Cl_2; (vii) S 270–275°C; (viii) $SOCl_2$; (ix) EtOH; (x) $h\nu$, I_2, O_2, toluene; (xi) aq NaOH.

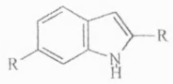
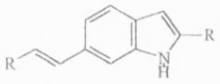
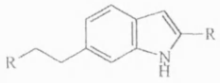
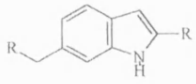
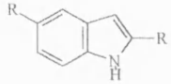
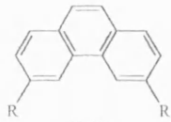
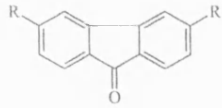
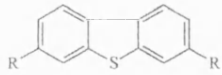
DDQ[15] to give 3,6-bisbromomethylphenanthrene **17** (40%). The required dicarboxylic acid **13** was obtained by sequential oxidation firstly with DMSO[16] to give the dialdehyde and secondly with chromium (VI)[17] (25% over 2 steps). The corresponding 3,6-dihydrophenanthrene dicarboxylate **14** was obtained in a separate synthesis by acetylation of dihydrophenanthrene itself under Friedel–Crafts conditions[18] to give the bismethylketone (24%) followed by oxidation to give the required diacid **14** and iodoform[19] (96%). The isomeric phenanthrene-2,7-dicarboxylic acid **15a** was prepared via pyrolysis of terephthalic acid in the presence of sulphur 270–275°C[20] to give the *trans*-stilbene-4,4′-dicarboxylate (**18a**, 20%). Conversion into the corresponding diethyl ester **18b** was carried out in two steps via the acid chloride (28–44%) and ethanolysis (79–87%). Photolysis in toluene solution in the presence of iodine and air[21] afforded diethyl phenanthrene-2,7-dicarboxylate **15b** (32%) and hydrolysis with aqueous sodium hydroxide gave the required phenanthrene dicarboxylic acid **15a** (99%).

Some linkers that seemed promising designs proved to be synthetically inaccessible. For example an approach to bisbenzofuran 3,7-dicarboxylic acid failed when the cyclisation of the precursor dimethyldihydroxybiphenyl with polyphosphoric acid afforded a cyclic phosphodiester (69%, Scheme 2) as the main isolable product.

Coupling of Dimers with Linker

The slight differences in reactivity and solubility between the various linkers meant that no single optimal method was found for the coupling reactions. This was true both of the hydrogenation of the nitro group of the precursor dimer and of the coupling reaction itself. For example, conditions for hydrogenating the bisimidazole intermediate **6** were crucial; a mixture of methanol and DMF (6:2.5 %) as solvent and a temperature of 40°C was found to be necessary. The most common methods used HBTU in the presence of *N*-methylmorpholine (Scheme 3).[23] In the case of the bisimidazole **6** the yield of isolated coupled product was only 5% after purification which contrasts with a yield of over 60% in the case of the flexible indole linker **10c** and the bispyrrole intermediate **5a**. Provided that sufficient material had been obtained for assay in DNA binding, optimisation experiments were not carried out in these couplings. The most difficult linkers to couple were the fully conjugated phenanthrene derivatives, perhaps because of the lower reactivity

Table 4. Linkers synthesised and their abbreviations

Structure (R = CO_2H throughout)	Abbreviation	No.
	*ind1*	**10a**
	*ind2*	**10b**
	*ind3*	**10c**
	*ind4*	**10d**
	*ind5*	**10e**
	*phen*	**13**
	*H_2phen*	**14**
	*fluo*	**12**
	*dbthia*	**11**
	*tere*	
	*fuma*	
	*t-cycp*	

of the corresponding carboxylic acids. The head to head dimers prepared are summarised in (Table 5). All products were purified using preparative HPLC under gradient elution conditions (see Experimental) and satisfactory NMR and electrospray mass spectra were obtained.

Affinity of Products for DNA

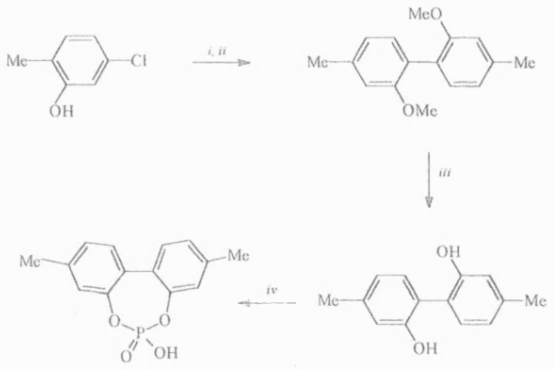
A small number of the compounds prepared have been evaluated in a preliminary study for their ability to bind to DNA;[7] we thank Dr Keith Fox (University of Southampton) for undertaking this study. Footprinting using TyrT DNA [24] as the target showed significant differences between the compounds studied. The fumaric acid linked compound **29** was studied in most detail; the results will be reported elsewhere.[7] The overall conclusion that can be drawn from the footprinting data is that these compounds are AT selective but cover larger regions than the parent antibiotic, distamycin. At moderate concentrations (~1 μM) there was evidence that they can span one or two CG base pairs. Using pAAD, pA_{3-6}, or pA/T_{3-12} DNA,[7] oligomers that offer a range of sizes of AT sites, the results suggested that more than four consecutive AT pairs are required to provide binding sites for these compounds. The tricyclic aromatic linkers, bisbenzothiophene **11** and dihydrophenanthrene **14**, gave compounds **26** and **19–25** with particularly strong binding at micromolar concentrations, principally to AT rich regions as would be expected. Similarly the indole 2,5-dicarboxylate derivative (**35** from **10e**) showed a strong footprint. On the other hand, 2,6-dicarboxylate analogues (**31–34** from **10a–d**) were comparatively poor binders. Although more detailed examination of these compounds is required, it is clear that the basic design strategy can lead to compounds of very high affinity for DNA. From the point of view of molecular design, compounds highly ranked such as **19–25** showed strong footprints but given the available binding data the modelling did not appear to have high predictive power. Moreover from the data available so far we cannot determine whether binding of the compounds containing polycyclic aromatic compounds involves intercalation. One of the benefits of using a polycyclic aromatic linker is that in principle, additional substituents can be added to modify solubility or to provide additional binding to DNA. Furthermore, it is possible to modify the synthetic routes to prepare asymmetric head to head linked compounds thereby expanding the ability to read defined sequences of DNA.

Experimental

The abbreviation used for each compound in the text and tables is given in the title line of each preparation in *italics*.

Instrumentation

Electrospray mass spectra (ES-MS) were obtained on a Fisons VG Platform Benchtop LC-MS. Electron impact (EI-MS) and fast atom bombardment (FAB-MS) mass spectra were obtained on a Jeol JMS-AX505HA mass spectrometer. NMR spectra were obtained on a Bruker AMX 400 spectrometer operating at 400 MHz for ^{1}H. In ^{1}H



Scheme 2. *Reagents:* (i) Me_2SO_4, NaOH; (ii) $NiCl_2ZnPPh_3$, α-dipy, DMF, 120°C; (iii) HBr/HOAc; (iv) polyphosphoric acid.

NMR spectra, the abbreviation 'exch.' signifies that the relevant resonances disappeared on treatment of the solution with D_2O.

HPLC purification of final products was carried out using a Vydac protein and peptide C18 column on a gradient eluting system. The solvents were *A*: water+0.1% trifluoroacetic acid, and *B*: acetonitrile 90%+water 10%+0.1% trifluoroacetic acid. The elution programme was as follows: (Table 6)

Preparation of monomers

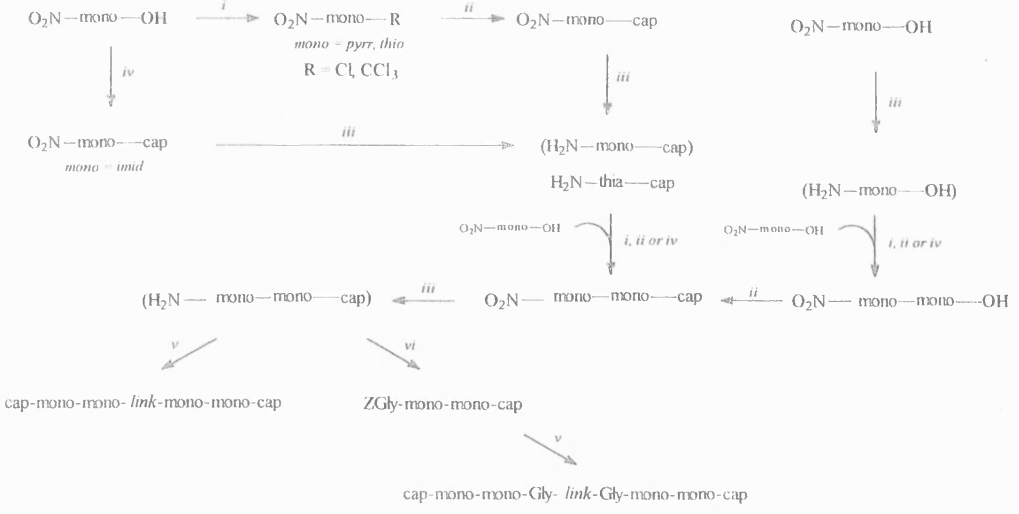
1-Methyl-4-nitropyrrole-2-carboxylic acid[4,5] **(1b).** (*O_2N pyrr OH*) This was prepared according to the literature[4,5] and obtained as a pale yellow powder (41% yield); mp 196–200°C (lit.[4] mp 204–205°C, lit.[6] mp 195–197°C).

1-Methyl-4-nitropyrrole-2-carbonyl chloride[10] **(1c).** (*O_2N pyrr Cl*) A standard literature procedure was used to give the product as grey solid material in quantitative yield, mp 90–91°C (lit.[10] mp 91–92°C). This acid chloride was used in the subsequent coupling without further purification.

1-Methyl-4-nitro-2-trichloroacetylpyrrole[9,10] **(1d).** (*O_2N pyrr CCl_3*) This was prepared in two steps from *N*-methylpyrrole by trichloroacetylation followed by nitration (96 and 41%, respectively) as pale yellow crystals mp 132–137°C (lit.[9] mp 135–140°C, lit.[10] mp 134–136°C).

3-(1-Methyl-4-nitropyrrole-2-carboxamido)dimethylaminopropane[11] **(1e).** (*O_2N pyrr dmap*) This was prepared using standard literature procedure. The product was obtained in (74% yield); mp 126–128°C (lit.[11] mp 126–127°C)

1-Methyl-4-(3-(1-methyl-4-nitropyrrole-2-carboxamido)-propyl)piperazine (1g). (*O_2N pyrr mpipp*) The pyrrole



Scheme 3. *Reagents:* (i) $SOCl_2$; (ii) amino or amidino cap, aq NaOH; (iii) H_2/Pd–C under solvent and temperature appropriate to each case (see Experimental); (iv) Me_3CCOCl, *N*-methylmorpholine, aminoacid cap; (v) HBTU, HOBT, *N*-methylmorpholine, *linker dicarboxylic acid*; (vi) HBTU, HOBT, ZglyOH.

Table 5. Head to head dimers synthesised

Compound	No.	Compound	No.
dmap pyrr pyrr H_2*phen* pyrr pyrr dmap	19	dmap pyrr pyrr *fuma* pyrr pyrr dmap	29
dheap pyrr pyrr H_2*phen* pyrr pyrr dheap	20	.....*t-cycp*.....	30
pyrp pyrr pyrr H_2*phen* pyrr pyrr pyrp	21	.....*ind1*.....	31
mpip pyrr pyrr H_2*phen* pyrr pyrr mpip	22	.....*ind2*.....	32
morphp pyrr pyrr H_2*phen* pyrr pyrr morphp	23	.....*ind3*.....	33
amidp pyrr pyrr H_2*phen* pyrr pyrr amidp	24	.....*ind4*.....	34
dmap pyrr pyrr *phen* pyrr pyrr dmap	25	.....*ind5*.....	35
.....*dbthi*.....	26	dmap imid imid H_2*phen* imid imid dmap	36
.....*fluo*.....	27	dmap thia pyrr H_2*phen* pyrr thia dmap	37
.....*tere*.....	28	dmap $(pyrr)_2$ Gly *phen* Gly $(pyrr)_2$ dmap	38

carboxylic acid **1c** (5.42 g, 31.9 mmol) was suspended in thionyl chloride (20 mL) and heated under reflux for 3 h. Excess thionyl chloride was removed under reduced pressure at room temperature then the remaining traces were co-evaporated with dichloromethane (30 mL, dry). *N*-methylpiperazine (5.00 g, 31.7 mmol) was diluted with dichloromethane (25 mL, dry) and added dropwise with stirring at 0°C to the solution of the acid chloride in dichloromethane (25 mL, dry). The reaction mixture was left stirring at room temperature overnight. The required *pyrrole carboxamide* precipitated as a yellow solid and filtered off, washed with dichloromethane (dry), and dried at 60°C under reduced pressure for 6 h (10.21 g, 93%.). Mp 185–188°C. ν_{max}/cm^{-1}(KBr) 1663, 1545, 1515, 1477, 1418, 1320. δ_H (DMSO-d_6): 1.72 (2H, s, CH_2); 2.51–3.26 (6H, m); 3.90 (3H, s, NMe pyrrole); 7.47 (1H, s, pyrrole); 8.13 (1H, s, pyrrole); 8.53 (1H, s, CONH exch.); 10.88 (1H, broad, HCl exch.). HRFABMS: Found: 310.18768 Calculated for $C_{14}H_{24}N_5O_3$ 310.18791.

4-(3-(1-Methyl-4-nitropyrrole-2-carboxamido)propyl)-morpholine (1h). (*O_2N pyrr morphp*) (a) *Acid chloride method:* A solution of **1c** (3.9 mmol: prepared as described earlier, from 2.36 g of the carboxylic acid **1b**), using 2.5 mL of thionyl chloride, and 10 mL of dimethoxyethane) in dry dimethoxyethane (20 mL) was cooled in an ice/water bath, and stirred while a solution of 4-(3-aminopropyl)morpholine (2.00 g, 13.9 mmol) in dry dimethoxyethane (5 mL) was added slowly during a period of 15 min. The resultant suspension was stirred at room temperature for 4 h, then diethyl ether (30 mL) was added. The solid was collected, washed with diethyl ether, and after drying under reduced pressure amounted to 3.88 g (84% yield of HCl salt of **1h**). This material was dissolved in water (100 mL) at about 50°C, and the solution was basified with 10% sodium carbonate (50 mL), and after cooling to 0°C, the precipitated solid was collected, and dried under reduced pressure to give the required *pyrrole carboxamide* (**1h**) as a fawn-coloured powder (0.875 g), mp 116–118°C; Found: C, 52.7; H, 7.05; N, 19.2% $C_{13}H_{20}N_5O_3$ requires: C, 52.69; H, 6.80; N, 18.91. ν_{max}/cm^{-1} (KBr) 3413, 3126, 2962, 2820, 1650, 1619, 1522, 1306, 1117; δ_H ($CDCl_3$) δ 1.79 (2H, m, CH_2–CH_2–CH_2), 2.54 (6H, m, 3×N–CH_2–CH_2), 3.50 (2H, q, J=6.0 Hz becoming t, J=6.2 Hz after D_2O, CONH–CH_2–CH_2), 3.77 (4H, t, J=4.5 Hz, CH_2–CH_2–O–CH_2–CH_2), 4.00 (3H, s, NCH_3), 7.10 (1H, d, J=1.6 Hz, Ar*H*), 7.55 (1H, d, J=1.6 Hz Ar*H*), 7.97 (1H, bs, exch., CON*H*). An additional 0.46 g of product (mp 112–116°C; IR identical to above) was obtained by extraction of the mother liquor with $CHCl_3$/methanol. The combined yield was 32%. (b) *Trichloroacetyl method:* A solution of 4-(3-aminopropyl)morpholine (2.93 g, 20.4 mmol) in dry THF (5 mL) was cooled in an ice/water bath, and stirred while a solution of 4-nitro-1-methyl-2-trichloroacetylpyrrole (**1d** 5.53 g, 20.4 mmol) in dry THF (15 mL) was added slowly during a period of 5 min. The solution was stirred at room temperature for 3 h, during which a solid separated out. Diethyl ether (40 mL) was added, and the solid was collected, washed with ether, and dried under reduced pressure to give the required *pyrrole carboxamide* (**1h**) (4.82 g, 80% yield) as a pale cream-coloured solid, mp 118–120°C. The mother liquor was evaporated under reduced pressure, and the residue was triturated with isopropanol to give additional sample (0.56 g, 9% yield) as a yellow solid, mp 118–120°C.

Table 6.

Step	Time (min)	Flow rate (mL min^{-1})	%*A*	%*B*
Equilibration	1	4	95	5
1	40	4	60	40
2	20	4	0	100
3	5	4	95	5
4	10	4	95	5

***N*-(3-(1-methyl-4-nitropyrrole-2-carboxamido)propyl)-diethanolamine (1i).** (*O_2N pyrr diheap*) A solution of *N*-(3-aminopropyl)-diethanolamine (500 mg, 3.086 mmol) in dry THF (2.0 mL) was cooled in an ice-water bath, and stirred while a solution of the trichloroacetyl pyrrole **1d** (837.5 mg, 3.086 mmol) in dry THF (2.0 mL) was added slowly over a period of 5 min. The mixture was stirred at room temperature for 20 h, then evaporated under reduced pressure to leave a golden yellow, syrupy residue. This was purified by flash column chomatography over silica, using a mixture of ethyl acetate (4 parts) and methanol containing 3% of conc. aq. ammonia solution (1 part) as eluant, to give the required *pyrrole carboxamide* **1i** as a yellow syrup (910 mg) which slowly crystallised. Recrystallisation from ethyl acetate/hexane gave pure **1i** as a pale yellow powder (594.8 mg, 61% yield), mp 80–82°C; ν_{max}/cm^{-1} (KBr) 3700–2500 (br), 3131, 2936, 2841, 1633, 1558, 1527, 1306, 1042; δ_H ($CDCl_3$) 1.78 (2H, m, CH_2–CH_2–CH_2), 2.64 (6H, m, 3×N–CH_2–CH_2), 3.50 (2H, q, J=4.6 Hz, becoming t, J=4.5 Hz after D_2O, NH–CH_2–CH_2), 3.67 (4H, t, J=4.6 Hz, 2×CH_2–CH_2-O), 3.98 (3H, s, N–CH_3), 7.23 (1H, d, J=1.6 Hz Ar*H*), 7.54 (1H, d, J=1.7 Hz Ar*H*);

(no signals were detected for N*H* or for 2×O*H*); ES-MS: 315.2; $C_{13}H_{22}N_4O_5$ requires 315.3 (M+1); HREIMS: Found: 314.15963 Calculated for $C_{13}H_{22}N_4O_5$ 314.15902.

3-(2-Carboxamido-1-methyl-4-nitroimidazole)dimethylaminopropane[24,25] (2b). (*O_2N imid dmap*) This was prepared using standard literature procedure. The product was obtained as yellow crystalline material in (60% yield); mp 140–142°C (lit.[24] mp 134°C; lit.[25] mp 210–211°C as HCl salt)

2-(5-Nitrothiophene-2-carboxamido)dimethylaminopropane (4b). (*O_2N thio dmap*) 5-Nitrothiophene 2-carboxylic acid (2.198 g, 12.69 mmol) was heated under reflux with thionyl chloride (10 mL) for 2 h, then the excess was removed under reduced pressure at 40°C. Dichloromethane (2×10 mL, dry) was added and removed under reduced pressure at 40°C. 3-Dimethylaminopropylamine (1.357 g, 13.28 mmol) was dissolved in dichloromethane (5 mL, dry) and added to a solution of the acid chloride in dichloromethane (25 mL, dry) dropwise, at 0°C with stirring. The yellow suspension that formed immediately was left stirring and allowed to warm to room temperature. The reaction mixture was extracted with water and the aqueous layer was basified with sodium carbonate. The yellow oil that precipitated was extracted with ethyl acetate, dried, and filtered, then the solvent was removed under reduced pressure at room temperature to give the required *thiophene carboxamide* as yellow crystals (2.21 g, 68% yield). Mp 102–104°C. (Found: C,46.6; H,5.9; N,16.2; S,12.3 $C_{10}H_{15}N_3O_3S$ requires C,46.7; H,5.9; N,16.3; S,12.5.) ν_{max}/cm^{-1} (KBr): 3336, 1628, 1563, 1534, 1514, 1355, 1340, 1320, 1302. δ_H (DMSO-d_6): 1.73–1.82 (2H, qt, *J*= 6.9 Hz, CH_2); 2.34 (6H, s, NMe_2); 2.57–2.59 (2H, t, 1.6 Hz, CH_2); 3.53–3.59 (2H, qt, *J*=4.5 Hz, CH_2); 7.29–7.31 (1H, d, *J*=1.5 Hz, ArH); 7.85–7.86 (1H, d, 1.5 Hz, ArH); 9.40 (1H, broad CONH exch.).

Preparation of dimers

3-[1-Methyl-4-(1-methyl-4-nitropyrrole-2-carboxamido)-pyrrole-2-carboxamido]dimethylaminopropane[11] (5a). (*O_2N pyrr pyrr dmap*) This was prepared using standard literature procedure. The product was obtained as pale yellow crystalline material in (79% yield); mp 190–192°C (lit.[11] mp 193–194°C).

1-Methyl-4-(3-(1-methyl-4-(1-methyl-4-nitropyrrole-2-carboxamido)pyrrole-2-carboxamido)propyl)piperazine (5c). (*O_2N pyrr pyrr mpipp*) The *N*-methylpiperazine amide hydrochloride **1g** (0.684 g, 1.966 mmol) was dissolved in ethanol (20 mL) and HCl (10 mL, dil). This solution was hydrogenated using Pd/C (256 mg, 10%) at room temperature and atmospheric pressure overnight. Filtration and washing the catalyst with water (10 mL) gave a pale yellow solution which was evaporated under reduced pressure at room temperature to give a pale yellow oil. This oil was dissolved in water (10 mL), to which was added gradually with stirring sodium hydrogen carbonate (1.305 g). The acid chloride was prepared from the carboxylic acid **1b** (406 mg, 2.39 mmol) heated under reflux in thionyl chloride (1 mL) and dimethoxyethane (5 mL, dry) for 2 h. The solvent was removed under reduced pressure at 40°C, then dissolved in benzene (10 mL), this solution was added dropwise with stirring at room temperature to the solution of **1g**. The resulting mixture was heated under reflux for 1/2 h. then left to cool to room temperature and stirring was continued overnight. The reaction mixture was extracted with dichloromethane, dried and solvent removed under reduced pressure to give yellow solid product (806 mg, 95%). This product was purified by flash column chomatography using silica gel and ethyl acetate/methanol/ammonium hydroxide (49%/49%/2%). TLC: R_f=0.2. Mp 175–177°C (from benzene/*n*-hexane). HREIMS: Found: 431.22612 Calculated for $C_{20}H_{29}N_7O_4$ 431.22810; ν_{max}/cm^{-1} (KBr): 1667, 1635, 1578, 1564, 1543, 1493, 1320. δ_H($CDCl_3$): 1.70–1.80 (2H, qt, *J*=6.9 Hz, CH_2); 2.29 (3H, s, NMe); 2.41–2.53 (10H, m); 3.43–3.50 (2H, q, *J*=6.1 Hz, CH_2); 3.91 (3H, s, NMe pyrrole); 4.04 (3H, s, NMe pyrrole); 6.66 (1H, d, *J*=1.7 Hz, pyrrole); 7.11 (1H, d, *J*=1.6 Hz, pyrrole); 7.26 and 7.27 (1H, d, *J*=1.6 Hz, pyrrole); 7.34–7.38 (1H, t, *J*=5.5 Hz, CONH exch.); 7.60 and 7.61 (1H, d, 1.8 Hz, pyrrole); 7.84 (1H, s, CONH exch.).

***N*-(3-(1-methyl-4-(1-methyl-4-nitropyrrole-2-carboxamido)-pyrrole-2-carboxamido)propyl)diethanolamine (5d).** (*O_2N pyrr pyrr dheap*) A mixture of the nitropyrrole amide (**1i**) (549 mg, 1.75 mol), 10% Pd–C (202 mg) and isopropanol (25 mL) was hydrogenated at room temperature for 5 h. The catalyst was removed by filtration through kieselguhr, and the filtrate was evaporated under reduced pressure. The green, oily residue was dissolved in dry DMF (2.0 mL), and the solution was cooled in an ice-water bath while a solution of the trichloroacetyl pyrrole **1d** (474 mg, 1.75 mmol) in dry DMF (1.0 mL) was added slowly during a period of 5 min. The resultant solution was stirred at room temperature for 16 h, then it was chomatographed over a flash column of silica, using a mixture of ethyl acetate (4 parts) and methanol containing 3% of conc. ammonium hydroxide (1 part) as eluant, to afford the required *amide* **5d** as an amber gum (307 mg, 40% yield). HRFABMS: Found: 437.21360 Calculated for $C_{19}H_{29}N_6O_6$ 437.21485; δ_H ($CDCl_3$) 1.74 (2H, m, CH_2–C*H*$_2$–CH_2), 2.67 (6H, m, 3×N–C*H*$_2$–CH_2), 3.43 (2H, q, 6.1 Hz, becoming t, *J*=3.5 Hz after D_2O, NH–CH_2–C*H*$_2$), 3.67 (4H, t, *J*= 3.5 Hz, 2×CH_2–C*H*$_2$–OH), 3.86 (3H, s, NMe), 4.01 (3H, s, NMe), 6.71 (1H, d, *J*=1.6 Hz, Ar*H*), 7.08 (1H, t, *J*= 5.5 Hz, exch., CON*H*–CH_2), 7.17 (1H, d, *J*=1.6 Hz, Ar*H*), 7.42 (1H, d, *J*=1.6 Hz, Ar*H*), 7.57 (1H, d, *J*= 1.6 Hz, Ar*H*), 8.92 (1H, s, exch., CON*H*).

4-(3-(1-Methyl-4-(1-methyl-4-nitropyrrole-2-carboxamido)-pyrrole-2-carboxamido)propyl)morpholine (5e). (*O_2N pyrr pyrr morphp*) A mixture of the nitropyrrole morpholine amide **1h** (1.30 g, 4.39 mmol.) and 10% Pd–C (510 mg) in isopropanol (50 mL) was hydrogenated at room temperature for 5 h. The catalyst was removed by filtration though kieselguhr, and the filtrate was evaporated under reduced pressure. Traces of isopropanol were removed from the residue by co-evaporation with dimethoxyethane (5 mL), to leave an amber oil. This oil was dissolved in dry dimethoxyethane (5 mL), and the solution was cooled in an ice/water bath, and stirred while a solution of the trichloroacetyl pyrrole **1d** (1.19 g, 4.39 mmol.) in a mixture of dry dimethoxyethane (2 mL) and dry THF (1.5 mL) was slowly added over a period of 5 min. The resultant solution

was stirred at room temperature for 16 h, then evaporated under reduced pressure. The semisolid residue was triturated with isopropanol until it solidified. The solid was collected, washed with isopropanol (5 mL) and dried under reduced pressure to give the required *amide* **5e** as a yellow powder (1.09 g, 59%), mp 182–184°C. HREIMS: Found: 418.19716 Calculated for $C_{19}H_{26}N_6O_6$ 418.19647; ν_{max}/cm^{-1} (KBr) 3408, 3125, 1656, 1634, 1574, 1535, 1502, 1309, 1116, 1110; δ_H (DMSO-d_6): 1.57–1.69 (2H, qt, J=7.0 Hz); 2.27–2.30 (6H, m); 3.15–3.27 (2H, q, J=6.0 Hz); 3.55–3.59 (4H, t, J=4.6 Hz); 3.80 (3H, s, NMe); 3.95 (3H, s, NMe); 6.83–6.84 (1H, d, J=1.7 Hz); 7.19–7.20 (1H, d, J=1.6 Hz); 7.56–7.57 (1H, d, J=1.8 Hz); 8.05–8.09 (1H, t, J=5.6 Hz, CONH exch.); 8.17–8.18 (1H, d, J=1.8 Hz); 10.23 (1H, s CONH exch.).

3-[1-Methyl-4-(1-methyl-4-nitropyrrole-2-carboxamido)-pyrrole-2-carboxamido]propionitrile[4,13] (5g). (*O_2N pyrr pyrr cyanp*) This was prepared using standard literature procedure. The product was obtained as yellow powder in (87% yield); mp 228–230°C (lit.[4] mp 230–232°C; lit.[13] mp 254–255°C).

Ethyl 3-[1-methyl-4-(1-methyl-4-nitropyrrole-2-carboxamido)pyrrole-2-carboxamido]propionimidate hydrochloride (5h). (*O_2N pyrr pyrr imidp*) A suspension of the foregoing nitrile **5g** (6.44 g) in anhydrous ethanol (250 mL) was cooled and stirred, and saturated at −20°C with dry HCl(g). The resultant mixture was stirred at room temperature for 3 h, then evaporated under reduced pressure. The residue was triturated with dry diethyl ether, decanted and dried under reduced pressure to give the required imidate **5h** (7.93 g, 99% yield) as a yellow solid; mp 134°C (decomp.). This was used in the next step without further purification.

3-[1-Methyl-4-(1-methyl-4-nitropyrrole-2-carboxamido)-pyrrole-2-carboxamido]propionamidine hydrochloride[4,11,12] (5f). (*O_2N pyrr pyrr amidp*) A suspension of the above imidate **5h** (3.345 g) in anhydrous ethanol (100 mL) was stirred and cooled to −78°C in an acetone–dry ice bath. Liquid ammonia (75 mL) was added, and the resultant mixture was allowed to warm up slowly to room temperature. A clear solution formed, from which a solid separated overnight. The precipitate was collected, washed with ethanol, then with diethyl ether, and dried under reduced pressure to give the required amidine (2.30 g, 74% yield) as a yellow powder; mp 308°C (decomp.). [Lit.[4] mp 315°C (decomp.), lit.[12] mp 324–325°C]. A further sample was prepared following the literature[11] in 69% yield mp 314–315°C [lit.[11] mp 246°C]. (Found: C, 54.4; H, 6.15; N, 19.9%; $C_{19}H_{26}N_6O$ requires: C, 54.54; H, 6.26; N, 20.08%.) δ_H (DMSO-d_6) 1.64 (2H, m, CH_2–CH_2–CH_2), 2.33 (6H, m, 3×N–CH_2–CH_2), 3.20 (2H, q, J=6.2 Hz becoming t, J=6.1 Hz, after D_2O, CONH–CH_2–CH_2), 3.57 (4H, t, J=4.7 Hz, CH_2–CH_2–O–CH_2–CH_2), 3.80 (3H, s, pyrrole NMe), 3.95 (3H, s, pyrrole NMe), 6.84 (1H, d, J=1.7 Hz, Ar*H*), 7.20 (1H, d, J=1.8 Hz, Ar*H*), 8.07 (1H, t, J=5.5 Hz, exch., CON*H*–CH_2), 8.18 (1H, d, J=1.7 Hz, Ar*H*), 10.24 (1H, s, exch., CON*H*).

3-{Carbobenzoxyglycyl-[1-methyl-4-(4-amino-1-methyl-pyrrole-2-carboxamido)pyrrole-2-carboxamido]} dimethylaminopropane (5i). (*Z Gly pyrr pyrr dmap*) A mixture of the nitropyrrole dimer **5a** (*O_2N pyrr pyrr dmap* 750 mg) and 10% Pd–C (690 mg) in isopropanol (75 mL) was hydrogenated at room temperature for 5 h. The catalyst was removed by filtration under an atmosphere of nitrogen, and the filtrate was evaporated under reduced pressure to leave crude amine as a greenish grey, glassy residue (631 mg, 91% yield), which was used immediately without further purification.

A mixture of carbobenzoxyglycine (457 mg, 2.19 mmol), HBTU (829 mg, 2.19 mmol), NMM (725 μL, =663 mg, 6.56 mmol.), and dry DMF (2.5 mL) was placed under nitrogen and stirred at room temperature for 30 min. To the resultant, clear solution was added a solution of the crude amine (631 mg, 1.82 mmol) in dry DMF (4 mL) slowly over a period of 5 min. The solution was stirred at room temperature for 15 h, then it was added to ethyl acetate (200 mL), extracted with 5% aqueous sodium carbonate solution (50 mL), washed with brine, and dried (Na_2SO_4) then evaporated under reduced pressure. The oily residue was dissolved in methanol, and the solution was passed though a short column of neutral alumina using methanol to elute the product which was used directly for the following preparation.

3-{Glycyl-[1-methyl-4-(4-amino-1-methylpyrrole-2-carboxamido)pyrrole-2-carboxamido]}dimethylaminopropane (5j). (*Gly pyrr pyrr dmap*) A mixture of foregoing trimer and 10% Pd–C (300 mg) in isopropanol (30 mL) was hydrogenated at 60°C for 15 h. The catalyst was removed by filtration though kieselguhr, and the filtrate was evaporated under reduced pressure to leave the deprotected trimer as an amber, gummy residue (196 mg, 82%); HPLC showed the material to be 90% pure and no unreacted starting material was detected. (Found ES-MS; 403.77, 201.46: $C_{19}H_{29}N_7O_3$ requires 404.5 (M+1), 202.75 [(M+2)/2]. The crude product was used immediately without further purification for the preparation of compound **38** below.

3-[1-Methyl-4-(1-methyl-4-nitroimidazol-2-carboxamido)-imidazol-2-carboxamido]dimethylaminopropane[25] (6). (*O_2N imid imid dmap*) This was prepared using standard literature procedure. The product was obtained as yellow solid in (58% yield); mp 160–161°C as HCl salt (lit.[25] mp 161–165°C as HCl salt).

3-[5-(5-Nitrothiophene-2-carboxamido)thiophene-2-carboxamido]dimethylaminopropane (7). (*O_2N thio thio dmap*) 3-(5-Nitrothiophene-2-carboxamido)dimethylaminopropane **4c** (226 mg, 0.879 mmol) and Pd/C (293 mg, 10%) were suspended in isopropanol (25 mL) and hydrogenated for 5 h. The reaction mixture was filtered through kieselguhr under nitrogen and the solvent was removed under reduced pressure. The acid chloride was prepared from 5-nitrothiophene-2-carboxylic acid **4a** (212 mg, 1.22 mmol) by heating under reflux for 3 h with thionyl chloride (3 mL). The excess thionyl chloride was removed under reduced pressure and the traces of thionyl chloride were removed by co-evaporation with dichloromethane. The amine produced by hydrogenation was dissolved in dichloromethane (3 mL, dry) and added dropwise at 0°C to a solution of the acid chloride in

dichloromethane (2 mL, dry). The reaction mixture was left stirring at room temperature overnight. The solvent was removed under reduced pressure and the yellow residual solid suspended in brine and extracted with ethyl acetate. The aqueous layer was basified with aq. sodium carbonate solution then extracted with ethyl acetate, the organic layer dried ($MgSO_4$) and the solvent removed under reduced pressure to give the required *amide dimer* (199 mg, 59% yield) as a dark brown solid, mp 185–190°C (softening). HRFABMS: Found: 383.08482 Calculated for $C_{15}H_{19}N_4O_4S_2$ 383.08477; ν_{max}/cm^{-1} (KBr) 1657, 1623, 1536, 1502, 1462, 1334, 1300. δ_H (DMSO-d_6): 1.64–1.76 (2H, qt, J=7.1 Hz, CH_2); 2.49–2.51 (2H, t, J=4.7 Hz, CH_2); 3.17–3.28 (2H, q, J=6.1 Hz, CH_2); 6.82 and 6.84 (1H, d, J=2.1 Hz, thiophene H); 7.22 and 7.24 (1H, d, J=2.1 Hz, thiophene H); 7.91 and 7.93 (1H, d, J=2.1 Hz, thiophene H); 8.17 and 8.18 (1H, d, J=2.1 Hz, thiophene H); 8.33–8.37 (1H, t, J=5.7 Hz, CONH exch.).

3-[4-Methyl-2-[(1-methyl-4-nitropyrrole-2-carboxamido)-thiazole-5-carboxamido](*N,N*-dimethyl)propanamine (8). (*O_2N pyrr thia dmap*) 4-Methyl-2-(1-methyl-4-nitropyrrole-2-carboxamido)thiazole-5-carboxylic acid (**3a**) (0.400 g, 1.289 mmol) was dissolved in benzene (40 mL, dry) and thionyl chloride (3 mL, freshly distilled) and heated under reflux for 4 h. The solvent was removed under reduced pressure, and the residue was co-evaporated with hexane (25×3 mL, dry). The product was used without any further purification. A cooled solution of *N,N*-dimethylaminopropylamine (350 μL, 2.781 mmol) and triethylamine (1 mL) in dichloromethane (20 mL, dry) was added dropwise to the above acid chloride in THF (25 mL, dry) under nitrogen. After the reaction mixture was stirred at room temperature for 12 h, the solvent was removed under reduced pressure and the resulting residue was extracted in a 1:1 mixture of THF:ethyl acetate (2×100 mL) and dil. solution of sodium bicarbonate. The organic layer was washed with brine, dried, and evaporated under reduced pressure at room temperature. The crude product was purified on a column of silica gel (methanol/ethyl acetate 1:1). The second fraction was collected and on evaporation afforded the required *amide* **8** (255 mg, 50%) as yellow crystals, mp 204–206°C. R_f=0.15 (silica, 1:1 methanol:ethyl acetate). HRFABMS: Found: 395.15210 Calculated for $C_{16}H_{23}N_6O_4S$ 395.15015; ν_{max}/cm^{-1} (KBr) 1625, 1502, 1430, 1430, 1378, 1316. δ_H (DMSO-d_6) 1.55–1.65 (2H, qt, J=7.1 Hz, CH_2); 2.15–2.25 (6H, s, NMe_2); 2.26–2.39 (2H, t, 4.6 Hz, CH_2); 2.50 (3H, s, thiazole-Me); 3.15–3.28 (2H, q, J=5.9 Hz, CH_2); 3.95–4.50 (3H, s, pyrrole-Me); 7.75–7.90 (1H, d, 2.2 Hz, ArH); 8.02–8.06 (1H, t, J=5.7 Hz, NH, exch.); 8.26 (1H, s, ArH).

3-[5-(4-Nitro-*N*-methylpyrrole-2-carboxamido)thiophene-2-carboxamido]dimethylaminopropane (9). (*O_2N pyrr thio dmap*) 4-Nitro-*N*-methylpyrrole-2-carboxylic acid **1b** (190 mg, 1.117 mmol) was suspended in thionyl chloride (4 mL) and heated under reflux for 1.5 h. Excess thionyl chloride was removed under reduced pressure and the last traces of it were removed by co-evaporation with dichloromethane (2×5 mL, dry). 2-(5-Nitrothiophene-2-carboxamido) dimethylaminopropane **4c** (205 mg, 0.797 mmol) and Pd/C (207 mg, 10%) were suspended in isopropanol (25 mL) and hydrogenated at room temperature and atmospheric pressure for 3 h. The catalyst was removed by filtration over kieselguhr under nitrogen, then the solvent was removed under reduced pressure at 40°C. The amine so formed was dissolved in dichloromethane (10 mL, dry) to which was added the acid chloride **1c** dissolved in dichloromethane (10 mL, dry) dropwise at 0°C. Stirring was continued overnight while the temperature was left to rise to room temperature The product precipitated as a yellow solid; it was filtered off, and washed with a small amount of dichloromethane (dry) to give the required *amide* **9** (163 mg, 50%). Mp>230°C. Some of this material was further purified by HPLC for characterisation. HRFABMS: Found: 380.13854 Calculated for $C_{16}H_{22}N_5O_4S$ 380.13925; ν_{max}/cm^{-1} (KBr): 1670, 1641, 1624, 1533, 1459, 1310, 1288. δ_H (DMSO-d_6): 1.84–1.88 (2H, qt, J=7.1 Hz, CH_2); 2.79 (6H, s, NMe_2); 3.08 (2H, t, J=4.7 Hz, CH_2); 3.08–3.29 (2H, q, J=6.2 Hz, CH_2); 3.98 (3H, s, NMe pyrrole); 6.84 and 6.85 (1H, d, 2.1 Hz); 7.54 and 7.55 (1H, d, J=2.0 Hz); 7.73 and 7.74 (1H, d, J=1.9 Hz); 8.28 and 8.28 (1H, d, 1.8 Hz); 8.43–8.46 (1H, t, J=5.5 Hz, CONH exch); 9.31 (1H, broad TFA exch); 11.65 (1H, s, CONH exch.).

Preparation of linkers

2,7-Bis(bromomethyl)-9,10-dihydrophenanthrene[15,16] (16). Literature procedures[15,16] were adapted as follows. A mixture of 9,10-dihydrophenanthene (20.11 g), paraformaldehyde (14.72 g), 85% phosphoric acid (22 mL), 48% aq. hydrogen bromide (38.5 mL), and 30% hydrogen bromide in acetic acid (44 mL) was placed under nitrogen and stirred and heated at 80°C for 21 h, then at 117°C (gentle reflux) for 5 h., and allowed to cool. The crude product separated as a grey solid mass. The supernatant liquid was decanted and the solid stirred with acetone (100 mL) at room temperature for 18 h, then cooled to 0°C. The insoluble solid was collected, washed with cold acetone, and recrystallised from benzene/petroleum ether (bp 40–60°C) to give the required dibromodihydrophenanthrene **16** as an off-white powder (16.23 g, 40% yield); mp 150–154°C (lit.[15] mp 157–158°C, lit.[16] mp 150–151°C); ν_{max}/cm^{-1} (KBr) 3414, 2925, 2896, 2830, 1640, 1618, 1488, 1243, 1205 cm^{-1}. δ_H ($CDCl_3$) 2.87 (4H, s, CH_2–CH_2), 4.53 (4H, s, 2×Ar–CH_2–Br), 7.28 (2H, s, Ar*H*), 7.33 (2H, d, J=8.0 Hz, Ar*H*), 7.71 (2H, d, 8.0 Hz, Ar*H*).

2,7-Diacetyl-9,10-dihydrophenanthene.[17,19] Literature procedures[17,19] for the Friedel–Crafts acetylation of 9,10-dihydrophenanthene were adapted as follows. Acetyl chloride (9.15 g, 117 mmol) was added with stirring using a motor-driven paddle stirrer to a suspension of aluminium trichloride (15.56 g, 117 mmol) in dry dichloromethane (75 mL), and the resultant solution was heated in a bath at a controlled temperature of 40°C, keeping the solvent just below the point of reflux. A solution of 9,10-dihydrophenanthene (9.54 g, 53.0 mmol) in dry dichloromethane (35 mL) was added dropwise over a period of 2 h, keeping the solvent just below refluxing temperature. Stirring was continued for a further 1 h at 40°C, then for 18 h at room temperature. The mixture was cooled in ice, and a solution of HCl (20 mL conc. HCl diluted with 30 mL water) was added slowly with vigorous stirring. An additional 50 mL of water was introduced to overcome emulsification and the layers were allowed to separate. The organic layer was

washed with 10% aq. sodium carbonate (250 mL), then with brine (100 mL), then dried (Na_2SO_4), and evaporated to leave an oil (15.25 g) which solidified slowly. This residue was stirred with acetone (20 mL) at room temperature for 1 h, and the solid was filtered off and washed with a little cold (−15°C) acetone. This crude product (7.78 g) was recrystallised from hot ethanol (100 mL) to afford 3.91 g of partially purified product, mp 138–142°C. Further recrystallisation from ethanol (60 mL) afforded the required diacetyldihydrophenanthrene, as golden yellow glistening plates (3.36 g, 24%) mp 142–144°C (lit.[17] mp 144–144.5°C); ν_{max}/cm^{-1} (KBr) 1681, 1677, 1641, 1603, 1262; δ_H ($CDCl_3$) 2.64 (6H, s, 2×C*H*$_3$CO), 2.98 (4H, s, C*H*$_2$–C*H*$_2$), 7.82–7.97 (6H, m, Ar*H*).

9,10-Dihydrophenanthrene-2,7-dicarboxylic acid[17] (14). The foregoing diketone was oxidised with iodine via its bispyridinium salt according to a literature procedure,[17] to give the crude diacid (**14**) as a dark brown powder (96% mp>350°C). Recrystallisation from aqueous DMF (decolourising charcoal) gave a lighter brown product, mp>360°C (lit.[17] mp~350°C); ν_{max}/cm^{-1} (KBr) 1686, 1640, 1613, 834; δ_H (DMSO-d_6) 2.90 (4H, s, C*H*$_2$–C*H*$_2$), 7.87–8.01 (6H, m, Ar*H*)

2,7-Bis(bromomethyl)phenanthrene (17). A mixture of the bisbromomethyldihydrophenanthrene (**16**, 4.62 g, 12.6 mmol), DDQ (3.15 g) and dry benzene (30 mL) was heated under reflux for 18 h. The mixture was filtered while still hot though a 2.5 in. column of neutral alumina, rinsing with hot benzene. The filtrate was evaporated under reduced pressure, and the residue was crystallised from benzene/petroleum ether (bp 40–60°C) to give the required *bisbromomethylphenanthrene* as pale buff-coloured crystals (2.94 g, 64% yield); mp 189–191°C. (Found: C, 52.9; H, 3.3; Br, 43.7%. $C_{16}H_{12}Br_2$ requires C, 52.78; H, 3.32; Br, 43.90%). ν_{max}/cm^{-1} (KBr) 1638, 1260, 1212, 1202, 906, 811 cm^{-1}; δ_H ($CDCl_3$) 4.73 (4H, s, 2×ArC*H*$_2$Br), 7.68–7.74 (4H, m, 4×Ar*H*), 7.90 (2H, d, *J*=1.7 Hz, 2×Ar*H*), 8.65 (2H, d, *J*=8.6 Hz, 2×Ar*H*).

2,7-Diformylphenanthrene.[26] This was prepared using standard literature procedure. The crude product was purified by flash column chromatography (SiO_2/dichloromethane) to give the required diformylphenathrene as bright yellow crystals in (52% yield), mp >350°C; ν_{max}/cm^{-1} (KBr) 1690, 1639, 1617, 1575 cm^{-1}. δ_H ($CDCl_3$) 7.95 (2H, s, 2×Ar*H*), 8.16–8.21 (2H, dd, *J*=1.7 Hz and 8.6 Hz 2×Ar*H*), 8.42 (2H, d, *J*=1.6 Hz, 2×Ar*H*), 8.84 (2H, d, *J*=8.6 Hz, 2×Ar*H*), 10.25 (2H, s, 2×ArC*H*O).

Phenanthrene-2,7-dicarboxylic acid[27] (13). The reagent (bipy)H_2CrOCl_5 reagent was freshly prepared according to the literature procedure[20] as a brown powder (31% yield). A solution of the foregoing dialdehyde (516 mg, 2.20 mmol) in dry dichloromethane (50 mL) was placed under nitrogen, and stirred at room temperature. (Bipy)H_2CrOCl_5 (4.45 g, 11.02 mmol) was added, and the mixture was stirred at room temperature for 18 h, then evaporated under reduced pressure. The residue was extracted *cautiously* (CO_2!) with a solution of sodium hydrogen carbonate (10 g) in water (250 mL). Some insoluble material was removed by filtration through kieselguhr, and the filtrate was acidified *cautiously* (CO_2!) with conc. hydrochloric acid. The precipitated solid was collected, washed with water, and dried under reduced pressure (1.66 g of crude product). The crude product was dissolved in a solution of sodium hydrogen carbonate (2.0 g) in water (100 mL), and reprecipitated with conc. hydrochloric acid. After drying under reduced pressure, the crude, dark brown solid amounted to 0.88 g (~150% theoretical yield of the required diacid). For further purification, the diacid was converted into its dimethyl ester and the ester hydrolysed to the acid as follows. The above crude product was suspended in methanol (125 mL) and the mixture was saturated at room temperature with dry hydrogen chloride (g), and then heated under reflux for 23 h. The resultant clear, green solution was evaporated under reduced pressure. The residue was extracted with dichloromethane (20 mL), and the dichloromethane was decanted from insoluble gummy material. This dichloromethane extract was purified by means of flash column chromatography (SiO_2/dichloromethane) to give the dimethyl ester as a cream-coloured solid (149 mg, 25%), mp 182–186°C. (Found: C, 73.5; H, 4.68%. $C_{18}H_{14}O_4$ requires C, 73.46; H, 4.79.%) ν_{max}/cm^{-1} (KBr) 1728, 1638, 1617; δ_H ($CDCl_3$) 4.03 (6H, s, 2×CO_2CH_3), 7.87 (2H, s, 2×Ar*H*), 8.32 (2H, dd, *J*=1.7 Hz and *J*=8.6 Hz, 2×Ar*H*), 8.64 (2H, d, *J*=1.6 Hz, 2×Ar*H*), 8.76 (2H, d, *J*=8.6 Hz, 2×Ar*H*).

A suspension of the above dimethyl ester (143.3 mg, 0.487 mmol) in a mixture of ethanol (5 mL) and 20% aqueous sodium hydroxide solution (5 mL, 25.0 mmol) was heated under reflux for 1 h. Water (10 mL) was added, and refluxing was continued for a further 2 h. The resulting solution was evaporated under reduced pressure, and the residue was dissolved in water (50 mL). This solution was cooled in an ice bath, and acidified with conc. HCl. The precipitated solid was collected on a filter paper, washed with water, and dried under reduced pressure to give the required *phenanthrene dicarboxylic acid* (**13**) as a pale yellow powder (118 mg, 91%), mp >350°C (lit. [27] Mp 165–167°C); ν_{max}/cm^{-1} (KBr) 1692, 1686, 1654, 1639, 1617 cm^{-1}. δ_H (DMSO-d_6) δ 8.09 (2H, s, 2×Ar*H*), 8.22 (2H, dd, *J*=1.8 Hz and *J*=8.6 Hz 2×Ar*H*), 8.67 (2H, d, *J*=1.7 Hz, 2×Ar*H*), 8.99 (2H, d, *J*=8.7 Hz, 2×Ar*H*), 13.26 (2H, br, exch., 2×CO_2H).

Diethyl stilbene-4,4′-dicarboxylate[21] (18b). A mixture of 4-toluic acid (50 g, 368 mmol) and sulphur flowers (5.88 g, 184 mmol) was placed in a 1-litre conical flask, and stirred and heated on a sand tray at 260–275°C (just on the bp of the toluic acid) for 2 h. The resultant mixture was allowed to cool to about 140°C, and hot xylene (200 mL) was added. A reflux air-cooled condenser was fitted, and the mixture was boiled under reflux for 30 min., then filtered hot. The filter cake was boiled with dioxane (75 mL) for 30 min., then filtered, washed with hot dioxane (50 mL) and dried under reduced pressure to afford the crude dicarboxylic acid **18a** as a yellow powder (12.71 g). The crude product was taken up in a boiling solution of potassium hydroxide (7.0 g) in water (300 mL). Some insoluble yellow material was removed by filtration, and the filtrate was concentrated to 100 mL, then allowed to cool. The dipotassium salt crystallised out as yellow plates (11.86 g after drying). The dipotassium salt was taken up in boiling water (350 mL),

and the resultant solution was acidified while hot with conc. HCl (15 mL). The resultant precipitate was digested hot for 1.5 h, and after cooling the product was collected, washed with water, and dried under reduced pressure to give the required dicarboxylic acid **18a** as a pale yellow powder (9.09 g, 37% yield based on sulphur), mp >320°C (lit.[21] Mp 460°C, [sealed tube]).

Diethyl ester 18b.[21,22] The above dicarboxylic acid (8.623 g, 32.4 mmol) was refluxed with thionyl chloride (50 mL) for 20 h. The resultant mixture was evaporated under reduced pressure, and residual thionyl chloride was removed from the solid residue by co-evaporation with hexane (2×30 mL). Crystallisation from benzene gave the acid chloride as a bright yellow powder (2.79 g, 28% yield), mp 222–228°C (lit.[21] mp 228–232°C, lit.[22] mp 223–224°C). (*Note*: When this reaction was carried out on a smaller scale, but with a reflux period of just 15 h, the yield of acid chloride was improved to 44%.) The acid chloride (2.78 g) was refluxed with ethanol (100 mL) for 17 h. The solution was evaporated under reduced pressure, and the crystalline residue recrystallised from ethanol to give the required diester as pale yellow crystals (2.58 g, 87% yield), mp 129–133°C (lit.[21] mp 129.9–130°C, lit.[23] mp 130–131°C); δ_H ($CDCl_3$) 1.32 (6H, t, J=7.0 Hz, 2×CH_2–CH_3), 4.39 (4H, q, J=7.0 Hz, 2×O–CH_2–CH_3), 7.23 (2H, s, Ar–C*H*=C*H*–Ar), 7.59 (4H, m, Ar*H*), 8.06 (4H, m, Ar*H*).

Diethyl 3,6-phenanthrene dicarboxylate[21] **(15b).** A solution of diethyl 4,4′-stilbene dicarboxylate **18b** (0.5015 g, 1.546 mmol) in toluene (1 L) containing iodine (0.1240 g) was irradiated for 3 days using a medium-pressure mercury lamp (400 W) in a quartz tube while oxygen was slowly bubbled though the reaction mixture. After removal of the solvent under reduced pressure, the residue was taken up in dichloromethane and passed through a short column of silica gel. Concentration of the eluate followed by addition of methanol precipitated a white solid which, on recrystallization from a dichloromethane/methanol mixture, gave the required phenanthrene as white prisms (243 mg, 49% yield) mp 162–164°C (lit.[21] mp 164–166°C).

3,6-Phenanthrene dicarboxylic acid (15a). Diethyl 3,6-phenanthrene dicarboxylate (150 mg, 0.466 mmol) was suspended in a mixture of ethanol (5 mL) and aq. sodium hydroxide (1.0 g in water 15 mL, 25 mmol). The reaction mixture was heated under reflux for 3 h, then solvent was removed under reduced pressure. The residue was dissolved in water (25 mL), cooled in ice, and acidified with conc. hydrochloric acid to afford the required phenanthrene dicarboxylic acid as a white solid which was filtered, washed with water and dried (912 mg, 99%). Mp>250°C; ν_{max}/cm^{-1} (KBr) 2981, 2824, 2641, 1691, 1622, 1449, 1422, 1295, 1276; δ_H (DMSO-d_6): 8.08–8.23 (6H, m, ArH); 9.35 (2H, s, ArH); 13.25 (2H, s, 2×CO_2H); HREIMS: Found: 266.05877 Calculated for $C_{16}H_{10}O_4$ 266.05791.

Cyclopropane-*trans*-1,2-dicarboxylic acid.[12,13] Sodium hydroxide solution (1.015 g, 2.56 mmol in water 10 mL) was added with stirring to a solution of dimethyl *trans*-cyclopropane dicarboxylate (1.085 g, 6.86 mmol) in ethanol (5 mL). The clear solution was left standing at room temperature for 3 h then solvent was removed under reduced pressure at room temperature. Water was added and removed under reduced pressure at room temperature. Ether (25 mL) and water (50 mL) were added and the disodium salt was extracted. The organic layer was extracted again with water. The water extracts were combined and acidified with conc. hydrochloric acid (pH 2). Water was removed under reduced pressure at room temperature, then the white solid mass was triturated with ethyl acetate (100 mL, dry) and filtered. The filtrate was removed under reduced pressure at room temperature to give the required diacid as a white solid (0.885 g, 99% yield), mp 177–178°C. [lit.[12] mp 172–174°C; lit.[13] mp 175°C]. ν_{max}/cm^{-1} (KBr): 1695. δ_H(DMSO-d_6): 1.14–1.29 (2H, t, J=8.1 Hz); 1.85–1.91 (2H, t, J=7.5 Hz); 12.58 (2H, broad 2×CO_2H exch.).

Preparation of head to head linked dimers (target compounds)

9,10-Dihydrophenanthrene-2,7-dicarboxamido{3-[1-methyl-4-(4-amino-1-methyl-pyrrole-2-carboxamido)pyrrole-2-carboxamido]-dimethylaminopropane} (19). (*dmap pyrr pyrr H_2phen pyrr pyrr dmap*) The nitro dimer compound (**5a**, 554 mg) in isopropanol (50 mL) was hydrogenated at room temperature over 10% Pd–C (537 mg) for 4 h. The catalyst was removed by filtration through kieselguhr under an atmosphere of nitrogen, and the filtrate was evaporated under reduced pressure to give the crude amine as a pale grey coloured, brittle, glassy solid (424 mg, 83%). This material was dissolved in dry DMF (4.0 mL), and the resultant solution (containing ~100 mg per mL) was used immediately in the following coupling reactions:

Using HBTU/NMM: A mixture of dihydrophenathrene-2,7-dicarboxylic acid **14** (25.8 mg, 0.0963 mmol), HBTU (110 mg, 0.289 mmol), NMM (65 μL, 0.594 mmol) and dry DMF (1.5 mL) was placed under nitrogen and stirred at room temperature for 30 min. A 1.0 mL aliquot of the solution of the amine derived from **5a** (100 mg, 0.289 mmol) above was added, and the mixture was stirred at room temperature for 18 h. The product was isolated from the resultant solution using HPLC (see Experimental). The fractions containing the product were frozen immediately on collection, and then freeze dried to give required *linked oligomer* **19**, bis-TFA salt, as a cream-coloured solid (54% yield) which had no distinct mp (Found: ES-MS: 925.7, 462.8; $C_{50}H_{60}N_{12}O_6$ (free base) requires 926.10 (M+1), 463.56 [(M+2)/2]); δ_H (DMSO-d_6) δ 1.84 (4H, m, 2×CH_2–CH_2–CH_2), 2.77 (12H, s, 2×NMe_2), 2.97 (4H, s, CH_2–CH_2 bridge), 3.04 (4H, m, 2×CH_2–CH_2), 3.26 (4H, m, 2×CH_2–CH_2), 3.83 (6H, s, 2×pyrrole NCH_3), 3.89 (6H, s, 2×pyrrole NCH_3), 6.95 (2H, d, J=1.6 Hz, 2×pyrrole Ar*H*), 7.12 (2H, d, J=1.6 Hz, 2×pyrrole Ar*H*), 7.19 (2H, d, J=1.6 Hz, 2×pyrrole Ar*H*), 7.34 (2H, d, J=1.6 Hz, 2× pyrrole Ar*H*), 7.90 (2H, s, Ar*H*), 7.93 (2H,d, J=8.0 Hz, Ar*H*), 8.05 (2H, d, J=8.4 Hz, Ar*H*), 8.16 (2H, t, exch., 2×CON*H*–CH_2), 9.95 (2H, s, exch., 2×CON*H*), 10.37 (2H, s, exch., 2×CON*H*).

Using diisopropylcarbodiimide/HOBT: A 1.0 mL aliquot of the solution of the primary amine derived from **5a** (100 mg, 0.289 mmol) above was added to a mixture of the dihydrophenathrene dicarboxylic acid **14** (25.8 mg, 0.0963 mmol),

HOBT (26 mg, 0.1926 mmol) and dry DMF (1.0 mL). The resultant mixture was placed under nitrogen, and stirred and cooled in an ice bath. A solution of diisopropylcarbodiimide (54.5 mg, 0.4325 mmol) in dry DMF (0.5 mL) was added, and the resultant solution was allowed to come to room temperature. After 18 h, water (2 drops) was added. The required *linked oligomer* was isolated as described above (49% yield).

The following compounds were prepared similarly.

9,10-Dihydrophenanthrene-2,7-dicarboxamido{3-[1-methyl-4-(4-amino-1-methyl-pyrrole-2-carboxamido)pyrrole-2-carboxamido]-dihydroxyethylaminopropane} (20). (*dheap pyrr pyrr H_2phen pyrr pyrr dheap*) In 45% yield from **5d** and dihydrophenanthrene-2,7-dicarboxylic acid using HBTU coupling. (Found: ES-MS: 1046.1, 523.9 $C_{54}H_{68}N_{12}O_{10}$ requires (free base) 1046.2 (M+1) and 523.6 [(M+2)/2]); ν_{max}/cm^{-1} (KBr) 1682, 1640, 1585, 1432, 1270, 1202; δ_H (DMSO-d_6) δ 1.91 (4H, m, 2×CH_2–CH_2–CH_2), 2.97 (4H, s, Ar–CH_2–CH_2–Ar), 3.17–3.24 (16H, m, 8×CH_2), 3.75 (8H, m, 4×CH_2), 3.82 (6H, s, 2× pyrrole NMe), 3.88 (6H, s, 2×pyrrole NMe), 5.30 (4H, bs, exch., 4×O*H*), 6.94 (2H, d, *J*=1.6 Hz, 2×pyrrole Ar*H*), 7.13 (2H, d, *J*=1.6 Hz, 2×pyrrole Ar*H*), 7.18 (2H, d, *J*=1.6 Hz, 2×pyrrole Ar*H*), 7.34 (2H, d, *J*=1.6 Hz, 2×pyrrole Ar*H*), 7.90–7.94 (4H, m, 4×benzene Ar*H*), 8.04–8.06 (2H, m, 2×benzene Ar*H*), 8.15 (2H, t, *J*=5.6 Hz, exch., 2×CON*H*–CH_2), 9.07 (2H, bs, exch., 2×TFA), 9.95 (2H, s, exch., 2×CON*H*), 10.34 (2H, s, exch., 2×CON*H*).

9,10-Dihydrophenanthrene-2,7-dicarboxamido{3-[1-methyl-4-(4-amino-1-methyl-pyrrole-2-carboxamido)pyrrole-2-carboxamido]-*N*-pyrrolidinylpropane} (21). (*pyrp pyrr pyrr H_2phen pyrr pyrr pyrp*) In 65% yield from **5b** and dihydrophenanthrene 2,7-dicarboxylic acid using HBTU coupling. (ES-MS: found 978.0, and 488.94; $C_{54}H_{66}N_{12}O_6$ requires (M+1) 979.1 and [(M+2)/2] 489.59.); ν_{max}/cm^{-1} (KBr) 1679, 1648, 1535, 1439, 1266, 1201; δ_H (DMSO-d_6): 1.82–1.91 (8H, m, 4×CH_2); 2.02–2.04 (4H, m, 2×CH_2); 2.97–3.02 (8H, m and s, 2×CH_2 and dihydrophenanthrene 2×CH_2); 3.13–3.18 (4H, m, 2×CH_2); 3.24–3.27 (4H, m, 2×CH_2); 3.56–3.57 (4H, m, 2×CH_2); 3.82 (6H, s, 2×NMe pyrrole); 3.88 (6H, s, 2×NMe pyrrole); 6.96 and 6.97 (2H, d, *J*=1.6 Hz, pyrrole); 7.12 and 7.13 (2H, d, *J*=1.6 Hz, pyrrole); 7.17 and 7.18 (2H, d, *J*=1.6 Hz, pyrrole); 7.33 and 7.34 (2H, d, *J*=1.6 Hz, pyrrole); 7.90 (2H, s, ArH); 7.92 and 7.94 (2H, d, *J*=1.6 Hz, ArH); 8.09 and 8.07 (2H, d, *J*=1.6 Hz, ArH); 8.14–8.17 (2H, t, *J*=5.6 Hz, CONH exch.); 9.4 (2H, broad, TFA exch.); 9.95 (2H, s, 2×CONH exch.); 10.37 (2H, s, 2×CONH exch.).

9,10-Dihydrophenanthrene-2,7-dicarboxamido{3-[1-Methyl-4-(4-amino-1-methyl-pyrrole-2-carboxamido)pyrrole-2-carboxamido]-(4-methylpiperazinyl)propane} (22). (*mpip pyrr pyrr H_2phen pyrr pyrr mpip*) In 43% yield from **5c** and dihydrophenanthrene 2,7-dicarboxylate using HBTU coupling. (ES-MS: Found: 1036.1, 518.1 $C_{56}H_{70}N_{14}O_6$ requires (M+1) 1036.2 and [(M+2)/2] 518.6; ν_{max}/cm^{-1} (KBr) 1680, 1641, 1538, 1437, 1267, 1200; δ_H (DMSO-d_6): 1.77 (4H, m, 2×CH_2); 2.72 (6H, s, 2×NMe); 2.97 (4H, s, dihydro); 3.23–3.24 (4H, q, *J*=5.6 Hz, 2×CH_2); 3.53 (20H, broad); 3.82 (6H, s, 2×NMe pyrrole); 3.88 (6H, s, 2×NMe pyrrole); 6.95 (2H, s, pyrrole); 7.12 (2H, s, pyrrole); 7.16 (2H, s, pyrrole); 7.34 (2H, s, pyrrole); 7.90 (2H, s, ArH); 7.92 and 7.94 (2H, d, *J*=8.4 Hz, ArH); 8.04 and 8.06 (4H, d, *J*=8.4 Hz, ArH and t, *J*=5.4 Hz, CONH exch.); 9.94 (2H, s, CONH exch.); 10.36 (2H, s, CONH exch.).

9,10-Dihydrophenanthrene-2,7-dicarboxamido{3-[1-methyl-4-(4-amino-1-methyl-pyrrole-2-carboxamido) pyrrole-2-carboxamido]-morpholine} (23). (*morphp pyrr pyrr H_2phen pyrr pyrr morph*) In 67% yield from **5e** and dihydrophenanthrene-2,7-dicarboxylic acid **14** using HBTU coupling. (Found: ES-MS: 1010.0 and 505.0; $C_{54}H_{64}N_{12}O_8$ requires (free base) 1010.18 (M+1) and 505.60 [(M+2)/2]); ν_{max}/cm^{-1} (KBr) 1677, 1642, 1534, 1440, 1265, 1201; δ_H (DMSO-d_6) δ 1.89 (4H, m, 2×CH_2–CH_2–CH_2), 2.97 (4H, s, Ar–CH_2–CH_2–Ar), 2.97–3.14 (8H, m, 4×CH_2), 3.25 (4H, m, 2×CH_2), 3.40 (4H, m, 2×CH_2), 3.63 (4H, m, 2×CH_2), 3.83 (6H, s, 2×NMe), 3.88 (6H, s, 2×NMe), 3.98 (4H, m, 2×CH_2), 6.96 (2H, d, *J*=1.6 Hz, 2×Ar*H*), 7.12 (2H, d, *J*=1.6 Hz, 2×Ar*H*), 7.18 (2H, d, *J*=2.0 Hz, 2×Ar*H*), 7.34 (2H, d, *J*=2.0 Hz, 2×Ar*H*), 9.6–9.8 (2H, bs, exch., 2×TFA) 9.95 (2H, s, exch., 2×CON*H*), 10.37 (2H, s, exch., 2×CON*H*).

9,10-Dihydrophenanthrene-2,7-dicarboxamido{3-[1-methyl-4-(4-amino-1-methyl-pyrrole-2-carboxamido)pyrrole-2-carboxamido]-propanamidine} (24). (*amidp pyrr pyrr H_2phen pyrr pyrr amidp*) In 35% yield from **5f** and dihydrophenanthrene-2,7-dicarboxylic acid **14** using HBTU coupling. (Found: ES-MS: 895.86 and 447.84; $C_{46}H_{50}N_{14}O_6$ (free base) requires: 895.99 (M+1) and 448.50 [(M+2)/2]); ν_{max}/cm^{-1} (KBr) 1682, 1640, 1585, 1540, 1270, 1202, 1134; δ_H (DMSO-d_6) δ 2.61 (4H, t, 2×CH_2–CH_2–C NH_4^+), 2.97 (4H, s, CH_2–CH_2 bridge), 3.51 (4H, q, *J*=6.0 Hz, becoming t, *J*=6.2 Hz after D_2O, 2×NH–CH_2–CH_2), 3.82 (6H, s, 2×pyrrole NCH_3), 3.88 (6H, s, 2×pyrrole NCH_3), 6.97 (2H, d, *J*=1.6 Hz, 2×pyrrole Ar*H*), 7.12 (2H, d, *J*=1.6 Hz, 2×pyrrole Ar*H*), 7.18 (2H, d, *J*=1.6 Hz, 2×pyrrole Ar*H*), 7.34 (2H, d, *J*=1.6 Hz, 2× pyrrole Ar*H*), 7.90 (2H, s, 2×Ar*H*), 7.93 (2H, d, *J*=8.4 Hz, 2×Ar*H*), 8.05 (2H, d, *J*=8.4 Hz, 2×Ar*H*), 8.20 (2H, t, *J*=5.6 Hz, exch., 2×CON*H*–CH_2), 8.53 (4H, s, exch., amidine), 8.90 (4H, exch., amidine), 9.95 (2H, s, 2×CON*H*), 10.37 (2H, s, 2×CON*H*).

Phenanthrene-3,6-dicarboxamido{3-[1-methyl-4-(4-amino-1-methyl-pyrrole-2-carboxamido)pyrrole-2-carboxamido]-dimethylaminopropane} (25). (*dmap pyrr pyrr phen pyrr pyrr dmap*) In 69% yield from **5a** and phenanthrene-3,6-dicarboxylic acid **15a** using HBTU coupling. (Found: ES-MS: 924.2 and 462.9 $C_{50}H_{58}N_{12}O_6$ (free base) requires 924.1 (M+1) and 462.5 [(M+2)/2]); ν_{max}/cm^{-1} (KBr) 1682, 1649, 1535, 1270, 1201; δ_H (DMSO-d_6) 1.85 (4H, qt, *J*=8.0 Hz, 2×CH_2–CH_2–CH_2), 2.80 (12H, d, *J*=2.8 Hz, becoming s after D_2O, 2×$NH^+(CH_3)_2$), 3.08 (4H, m, becoming t, *J*=5.6 Hz after D_2O, 2×NH–CH_2–CH_2), 3.26 (4H, q, *J*=6.8 Hz, becoming t, *J*=5.6 Hz after D_2O, 2×NH–CH_2–CH_2), 3.83 (6H, s, 2×pyrrole NCH_3), 3.91 (6H, s, 2×pyrrole NCH_3), 6.97 (2H, d, *J*=1.6 Hz, 2×pyrrole Ar*H*), 7.18 (2H,d, 2×pyrrole Ar*H*), 7.19 (2H, d, *J*=1.6 Hz, 2×pyrrole Ar*H*), 7.40 (2H, d, *J*=1.6 Hz, 2× pyrrole Ar*H*), 8.06 (2H,s, Ar*H*), 8.17 (2H, t, *J*=5.6 Hz, exch.,

2×CONH–CH_2), 8.28 (2H,d, J=2.1 Hz, 2×ArH), 8.63 (2H, d, J=2.0 Hz, 2×ArH), 9.05 (2H, d, J=4.5 Hz, 2×ArH), 9.28 (2H, br, exch., 2×NH^+), 9.98 (2H, s, exch., 2×CONH), 10.60 (2H, s, exch., 2×CONH).

Using HATU/NMM: A mixture of **15a** (25.6 mg, 0.0963 mmol), HATU (115 mg, 0.303 mmol), NMM (65 μL, 0.594 mmol) and dry DMF (1.5 mL) was placed under nitrogen and stirred at room temperature for 30 min. A 1.0 mL aliquot of the solution of the primary amine derived from **5a** (100 mg, 0.289 mmol) above was then added, and the mixture was stirred at room temperature for 18 h. Yield 80.7 mg (73%).

Bisbenzothiophene-3,7-dicarboxamido{3-[1-methyl-4-(4-amino-1-methyl-pyrrole-2-carboxamido)pyrrole-2-carboxamido]-dimethylaminopropane} (26). (*dmap pyrr pyrr dbthi pyrr pyrr dmap*) In 62% yield from **5a** and bisbenzothiophene-3,7-dicarboxylic acid **11** using HBTU coupling. (Found: ES-MS: 929.8, 465.6; $C_{48}H_{56}N_{12}O_6S$ requires: (M+1) 929.85, [(M+2)/2] 464.9). δ_H (DMSO-d_6): 1.82–1.86 (4H, qt, J=6.8 Hz, 2×CH_2); 2.78 (12H, s, 2×NMe_2); 3.04–3.08 (4H, t, J=7.2 Hz, 2×CH_2); 3.23–3.32 (4H, q, J=6.8 Hz, 2×CH_2); 3.83 (6H, s, 2×NMe pyrrole); 3.90 (6H, s, 2×NMe pyrrole); 6.96 and 6.97 (2H, d, J=1.6 Hz); 7.15 (2H, d, J=1.6 Hz); 7.19 (2H,d, J=1.6 Hz); 7.37 (2H, d, J=1.6 Hz); 8.11 and 8.12 (2H, d, J=8.0 Hz); 8.14–8.18 (2H, t, J=6.0 Hz,CONH exch); 8.59 and 8.61 (2H, d, J=8.0 Hz); 8.65 (2H, s); 9.30 (2H, broad, 2×TFA exch.); 9.9 (2H, s, CONH exch); 10.54 (2H, s, CONH exch.).

Fluorenone-3,6-dicarboxamido{3-[1-methyl-4-(4-amino-1-methyl-pyrrole-2-carboxamido)pyrrole-2-carboxamido]-dimethylaminopropane} (27). (*dmap pyrr pyrr fluo pyrr pyrr dmap*) In 45% yield from **5a** and fluorenone-3,6-dicarboxylic acid **12** using HBTU coupling. (Found: ES-MS: 925.8, 462.7 $C_{49}H_{56}N_{12}O_7$ requires (M+1) 926.1; [(M+2)/2] 463.5). δ_H (DMSO-d_6): 1.81–11 (4H, qt, J=8.0 Hz, 2×CH_2); 2.79 (12H, s, 2×Me_2); 3.06–3.10 (4H, t, J=7.8 Hz, 2×CH_2); 3.23–3.26 (4H, q, J=6.4 Hz, CH_2); 3.83 (6H, s, 2×pyrrole-Me); 3.89 (6H, s, 2×pyrrole-Me); 6.96 and 6.96 (2H, d, J=1.6 Hz, ArH); 7.14 (2H, d, J=1.6 Hz, ArH); 7.18 and 7.19 (2H, d, J=1.6 Hz, ArH); 7.35and 7.36 (2H, d, J=1.6 Hz, ArH); 8.06 and 8.08 (2H, d, J=8.4 Hz, ArH); 8.14–8.17 (2H, t, J=8.4 Hz, 2×NH exch.); 8.27–8.28 (4H, m, ArH); 9.27 (2H, broad, TFA exch.); 9.97 (2H, s, 2×NH exch.); 10.56 (2H, s, 2×NH exch.).

1,4-Benzenedicarboxamido{3-[1-Methyl-4-(4-amino-1-methyl-pyrrole-2-carboxamido)pyrrole-2 carboxamido]-dimethylaminopropane} (28). (*dmap pyrr pyrr tere pyrr pyrr dmap*) In 34% yield from **5a** and terephthalic acid using HBTU coupling. (Found: ES-MS 824.0, 412.8; $C_{42}H_{54}N_{12}O_6$ requires: (M+1) 823.68, [(M+2)/2] 412.84); ν_{max}/cm^{-1} (KBr) 1680, 1642, 1538, 1267, 1200; δ_H (DMSO-d_6): 1.82–1.86 (4H, qt, J=8.0 Hz, 2×CH_2); 2.79 (6H, s, 2×NMe); 2.80 (6H, s, 2×NMe); 3.05–3.10 (4H, m, 2×CH_2); 3.22–3.27 (4H, m, 2×CH_2); 3.82 (6H, s, 2×NMe pyrrole); 3.88 (6H, s, NMe pyrrole); 6.95 and 6.96 (2H, d, J=2.0 Hz); 7.12 and 7.13 (2H, d, J=1.6 Hz); 7.18 (2H, d, J=1.6 Hz); 7.35 (2H, d, J=1.6 Hz); 8.06 (4H, s); 8.14–8.17 (2H, t, J=5.6 Hz, CONH exch.); 9.35 (2H, broad TFA exch.); 9.95 (2H, s, 2×CONH exch.); 10.47 (2H, s, 2×CONH exch.).

***Trans*-cyclopropane-1,2-dicarboxamido{3-[1-methyl-4-(4-amino-1-methyl-pyrrole-2-carboxamido)pyrrole-2 carboxamido]-dimethylaminopropane} (30).**[6] (*dmap pyrr pyrr cycp pyrr pyrr dmap*) In 46% yield from **5a** and *trans*-cyclopropane 1,2-dicarboxylic acid using HBTU coupling. (Found: ES-MS 787.0, 393.6. $C_{36}H_{54}N_{12}O_6$ requires (M+1) 787.65; [(M+2)/2] 394.5); ν_{max}/cm^{-1} (KBr) 1682, 1649, 1582, 1535, 1438, 1201; δ_H (DMSO-d_6): 1.21–1.24 (2H, t); 1.81–1.85 (4H, qt, J=7.9 Hz); 2.18–2.21 (2H, t, J=7.2 Hz); 2.77 (12H, s, 2×NMe_2); 3.03–3.07 (4H, t, J=8.0 Hz); 3.21–3.26 (4H, q, J=6.4 Hz); 3.81 (6H, s, 2×NMe pyrrole); 3.82 (6H, s, 2×NMe pyrrole); 6.85 and 6.86 (2H, d, J=2.0 Hz); 6.92 and 6.93 (2H, d, J=2.0 Hz); 7.15–7.17 (4H, dd, J=1.6 Hz and J=2.0 Hz); 8.12–8.15 (2H, t, J=5.6 Hz, CONH exch.); 9.50 (2H, broad TFA exch.); 9.86 (2H, s, CONH exch.); 10.24 (2H, s, CONH exch.).

9,10-Dihydrophenanthrene-2,7-dicarboxamido{3-[1-methyl-4-(4-amino-1-methyl-imidazol-2-carboxamido)imidazol-2-carboxamido]-dimethylaminopropane} (36). (*dmap imid imid H_2phen imid imid dmap*) In 5% yield from **6** and dihydrophenanthrene-2,7-dicarboxylic acid **14** using HBTU coupling. (Found: ES-MS: 929.7, 464.8 $C_{46}H_{57}N_{16}O_6$ requires (M+1) 930.1 and [(M+2)/2] 465.5); ν_{max}/cm^{-1} (KBr) 1677, 1641, 1582, 1405, 1265, 1200; δ_H (DMSO-d_6): 1.81–1.85 (4H, qt, J=3.6 Hz); 2.79 (6H, s, 2×NMe_2); 2.77 (6H, s, 2×NMe_2); 2.92 (4H, s); 3.03–3.07 (4H, t, J=18.8 Hz); 3.21–3.26 (4H, q, J=8.0 Hz); 3.97 (6H, s, 2×NMe_2 imidazole); 4.04 (6H, s, 2×NMe_2 imidazole); 7.56 (2H, d, J=4.8 Hz); 7.68 (2H, d, J=4.4 Hz); 7.96–8.07 (6H, m, ArH); 8.45–8.47 (2H, t, J=6.0 Hz, CONH exch.); 9.27 (2H, s, TFA exch.); 9.41 (2H, s, CONH exch.); 10.88 (2H, s, CONH exch.).

9,10-Dihydrophenanthrene-2,7-dicarboxamido{3-[2-(4-amino-1-methyl-pyrrole-2-carboxamido)thiazol-4-carboxamido]-dimethylaminopropane} (37). (*dmap thia pyrr H_2phen thia pyrr dmap*) In 40% yield from **8** and dihydrophenanthrene 2,7-dicarboxylic acid **14** using HBTU coupling. (Found: ES-MS: 962.01, 481.90. $C_{48}H_{56}O_6N_{12}S_2$ requires: (M+1) 962.20, [(M+2)/2] 481.60).); ν_{max}/cm^{-1} (KBr) 1680, 1647, 1538, 1397, 1203, 1130; δ_H (DMSO-d_6): 1.84–1.90 (4H, qt, J=6.7 Hz, 2×CH_2); 2.54 (6H, s, thiazole Me); 2.67 (6H, s, 2×NMe); 2.79 (6H, s, 2×NMe); 2.97 (4H, s, dihydrophenanthrene); 3.06–3.10 (4H, q, J=6.8 Hz, 2×CH_2); 3.11–3.27 (4H, m, 2×CH_2); 3.93 (6H, s, 2×NMe pyrrole); 7.45 (2H, s, pyrrole); 7.60 (2H, s, pyrrole); 7.92 (2H, s, ArH); 7.93 and 7.95 (2H, d, J=1.9 Hz, ArH); 8.05–8.11 (4H, s and t, J=4.8 Hz, ArH and {CONH exch.}); 9.24 (2H, broad, exch.); 10.47 (2H, s, CONH exch.); 12.48 (2H, s, CONH exch.).

Phenanthrene-3,6-dicarboxamido{3-[glycyl-1-methyl-4-(4-amino-1-methyl-pyrrole-2-carboxamido)pyrrole-2-carboxamido]-dimethylaminopropane} (38). (*dmap pyrr pyrr gly phen gly pyrr pyrr dmap*) In 49% yield from **5j** and dihydrophenanthrene-3,6-dicarboxylic acid **15a** using HBTU coupling. (Found: ES-MS: 1037.9 $C_{54}H_{64}N_{14}O_8$ requires (free base) (M+1) 1038.2 and [(M+2)/2]

2519.6); ν_{max}/cm^{-1} (KBr) 1678, 1647, 1582, 1534, 1266, 1201; δ_H (DMSO-d_6) 1.83 (4H, m, 2×CH_2–CH_2–CH_2), 2.78 (12H, d, J=2.8 Hz, becoming, s, after D_2O, 2×$NH^+[CH_3]_2$), 3.06 (4H, m, 2×N–CH_2–CH_2), 3.24 (4H, m, 2×N–CH_2–CH_2), 3.80 (6H, s, 2×pyrrole NMe), 3.83 (6H, s, 2×pyrrole NMe), 4.13 (4H, d, J=5.6 Hz, becoming, s, after D_2O, 2×NH–CH_2–CO), 6.92 (2H, d, J=1.6 Hz, 2×pyrrole Ar*H*), 6.95 (2H, d, J=1.6 Hz, 2×pyrrole Ar*H*), 7.15 (2H, d, J=1.6 Hz, 2×pyrrole Ar*H*), 7.19 (2H, d, J=1.6 Hz, 2×pyrrole Ar*H*), 8.04 (2H, s, Ar*H*), 8.16 (6H, m, becoming 4H after D_2O, 2×CON*H*CH_2 plus 4×Ar*H*), 9.24 (2H, t, J=5.6 Hz, exch., 2×CON*H*CH_2), 9.35 (2H, bs, exch., 2×TFA), 9.48 (2H,s, 2×Ar*H*), 9.86 (2H, s, exch., 2×CON*H*), 10.01 (2H, s, exch., 2×CON*H*).

References

1. Wemmer, D. E.; Dervan, P. B. *Curr. Opin. Struct. Biol.* **1997**, *7*, 355–361; Helene, C. *Nature* **1998**, *391*, 436–438.
2. Neidle, S. *Biopolymers* **1997**, *44*, 105–121; Johannesson, H, Halle, B. *J. Am. Chem. Soc.* **1998**, *120*, 6859–6870; Reddy, B. S.; Sondhi, S. M.; Lown, J. W. *Pharmacol. Therapeutics* **1999**, *84*, 1–111.
3. Walker, W. L.; Kopka, M. L.; Goodsell, D. S. *Biopolymers* **1998**, *44*, 323–334.
4. Dwyer, T. J.; Geierstanger, B. H.; Bathini, Y.; Lown, J. W.; Wenner, D. E. *J. Am. Chem. Soc.* **1992**, *114*, 5911–5919; Mrkisch, M.; Wade, W. S.; Dervan, P. B. *J. Am. Chem. Soc.* **1992**, *114*, 8783; Swalley, S. E.; Baird, E. E.; Dervan, P. B. *J. Am. Chem. Soc.* **1997**, *119*, 6953–6961; Turner, J. M.; Baird, E. E.; Dervan, P. B. *J. Am. Chem. Soc.* **1997**, *119*, 7636–7644; White, S.; Baird, E. E.; Dervan, P. B. *J. Am. Chem. Soc.* **1997**, *119*, 8756–8765; White, S. Turner, J. M., Szewczyk, J. W.; Baird, E. E.; Dervan, P. B. *J. Am. Chem. Soc.* **1999**, *121*, 260–261.
5. Herman, D. M.; Baird, E. E.; Dervan, P. D. *J. Am. Chem. Soc.* **1998**, *120*, 1382–1391; Turner, J. M.; Swalley, S. E.; Baird, E. E.; Dervan, P. B. *J. Am. Chem. Soc.* **1998**, *120*, 6219–6226.
6. Wang, W.; Lown, J. W. *J. Med. Chem.* **1992**, *35*, 2890–2897; Singh, M. P.; Plouvier, B.; Hill, G. C.; Gueck, J.; Pan, R. T.; Lown, J. W. *J. Am. Chem. Soc.* **1994**, *116*, 6995–7005; Kissinger, K. L.; Dabeowiak, J. C; Lown, J. W. *Chem. Res. Toxicol.* **1990**, *30*, 162–168; Rao, K. E.; Krowczki, K.; Balzarini, J.; DeClerq, E.; Newman, R. A.; Lown, J. W. *Actual Chim. Ther.* **1991**, *18*, 21–42.
7. Fishleigh, R. V.; Fox, K. R.; Khalaf, A. I.; Pitt, A. R.; Scobie, M.; Suckling, C. J.; Urwin, J.; Waigh, R. D., Young, S. C. *J. Med. Chem.* **2000**, in press.
8. Florence, A. T.; Attwood, D. *Physicochemical Principles of Pharmacy*, 3rd ed.; Macmillan: London, 1998; p 185.
9. Dodson R. M.; King, L. C. *J. Am. Chem. Soc.* **1945**, *67*, 2242–2243; Roa, K. E.; Bathini, Y.; Lown., J. W. *J. Org. Chem.* **1990**, *55*, 728–737.
10. (a) Glibin, E. N.; Tsukerman, B. V.; Ginsburg, O. F. *J. Org. Chem. USSR [Eng]* **1977**, *13*, 2082–2083; Glibin, E. N.; Tsukerman, B. V.; Ginsburg, O. F. *J. Org. Chem. USSR [Eng]* **1981**, *17*, 568–572. (b) Compound **2a** was obtained from Proteus International. (c) Compound **4a** was obtained from Proteus International.
11. Lown, J. W.; Krowicki, K. *J. Org. Chem.* **1985**, *50*, 3774–3779; Julia, M.; Preau-Joseph, N. *Compt. Rend. Acad. Sci. Fr.* **1963**, *257*, 1115.
12. Nishiwaki, E.; Tanaka, S.; Lee, H.; Shibuya, M. *Heterocycles* **1988**, *27*, 1945.
13. Khalaf, A. I.; Pitt, A. R.; Suckling, C. J.; Scobie, M.; Urwin, J.; Waigh, R. D., Fishleigh, R. V.; Young, S. C. *J. Chem. Res.* **2000**, in press.
14. Bisbenzothiophene was obtained from Proteus International; fluorenone dicarboxylic acid was obtained from Florochem.
15. Helms, A.; Heiler, D.; McLendon, G. *J. Am. Chem. Soc.* **1992**, *114*, 6227–6238.
16. Tashiro, M.; Nago, H.; Furusawa, T.; Tsuge, A.; Mataka, S.; Yamato, T. *Chem. Express* **1991**, *6*, 181–184.
17. Chakrabarty, T. K.; Chandrasekaran, S. *Synth. Commun.* **1980**, *10*, 951.
18. Paioni, R.; Jenny, W. *Helv. Chim. Acta* **1970**, *53*, 141–154.
19. Buu-Hoi, N. P.; Mabille, P.; Thang, D.-C. *Bull. Soc. Chim. Fr.* **1966**, 1667–1670.
20. Toland, W. G.; Wilkes, J. B.; Brutschy, F. J. *J. Am. Chem. Soc.* **1953**, *75*, 2263–2264.
21. DuVernet, R. B.; Wennerström, O.; Lawson, L.; Otsubo, T.; Boekelheide, V. *J. Am. Chem. Soc.* **1978**, *100*, 2457–2464.
22. Elix, J. A.; Kennedy, J. M. *Aust. J. Chem.* **1985**, *38*, 1857–1861; Giles, R. G. F.; Sargent, M. V. *Aust. J. Chem.* **1986**, *39*, 2177–2181.
23. Spencer, J. R.; Antonenko, V. V.; Delaet, N. C. J.; Goodman, M. *Int. J. Peptide Protein Res.* **1992**, *40*, 282–293; Knorr, R.; Trzeciak, A.; Bannwarth, W., Gillessen, D. *Tetrahedron* **1989**, *30*, 1297–1930; Carpino, L. A. *J. Am. Chem. Soc.* **1993**, *115*, 4397–4398.
24. Al-Said, N. H.; Lown, L. W. *Synth. Commun.* **1995**, *25*, 1059–1070.
25. Nishiwaki, E.; Lee, H.; Matsumoto, T.; Toyooka, K.; Sakurai, H.; Shibuya, M. *Tetrahedron Lett.* **1990**, *31*, 1299–1302.
26. Heiler, D.; McLendon, G.; Rogalskyi, P. *J. Am. Chem. Soc.* **1972**, *94*, 2414–2421.
27. Reiser, A.; Leyshon, L. J.; Saunders, D.; Mijovic, M. V. *J. Am. Chem. Soc.* **1972**, *94*, 2414–2421.

Synthesis of novel DNA binding agents: indole-containing analogues of bis-netropsin

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Molecular modelling studies showed that indole dicarboxylic acids are potential linkers for the synthesis of bis-netropsin analogues with a good fit to the minor groove of DNA. To test this hypothesis, 2-carboxyindole-6-acetic acid, indole-2,6-dicarboxylic acid, 6-(2-carboxyethyl)indole-2-carboxylic acid, 6-(2-carboxy-1-ethenyl)indole-2-carboxylic acid were prepared and coupled to 3-[1-methyl-4-(1-methyl-4-aminopyrrole-2-carboxamido)pyrrole-2-carboxamido]dimethylaminopropane. Similarly indole-2,5-dicarboxylic acid was prepared and coupled to 3-[1-methyl 4-(1-methyl-4-aminopyrrple-2-carboxamido)pyrrole-2-carboxamido]propionamidine hydrochloride. The derivatives of **26–28** showed especially strong binding to AT rich regions as shown by footprinting studies.

Oligomers of 3-amino-N-methyl-5-carboxylic acid such as the natural products distamycin and netropsin have cytotoxic properties because of their ability to bind to AT rich regions of the minor groove of DNA. In order to extend the available reading frame, several groups have prepared head to head linked dimers, known as bis-netropsins.[1–4] Detailed molecular modelling studies[7] showed that indole dicarboxylic acids would be good linking molecules. The selection of compounds for synthesis was made to include two substitution patterns and variation in length and rigidity of one of the side chains. This paper describes the synthesis of four new bis-netropsins of this type[9–11] the structures of which are shown in Fig. 1.

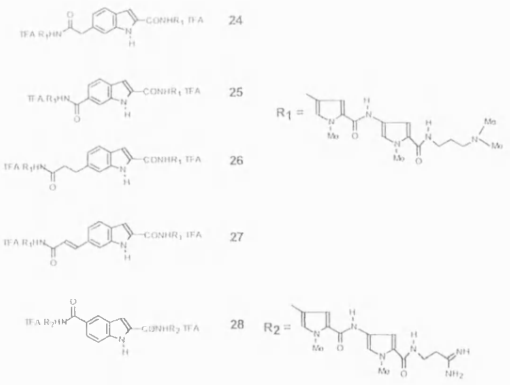
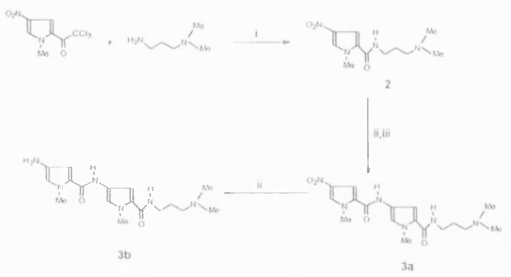


Fig. 1 Indole-linked head to head netropsin analogues prepared in this work

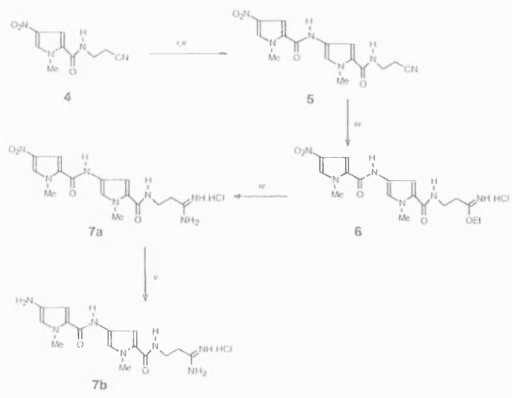
The pyrrole oligomers (R_1 and R_2, Fig. 1) were prepared by adaptations of published methods.[12–15] The indole dicarboxylic acids were obtained from side-chain modification of products of Fischer indole synthesis as shown in Schemes 3 and 4.

3a (Scheme 1) was hydrogenated in the presence of palladised charcoal at room temperature and atmospheric pressure to give the amine **3b** which was both air and light sensitive. Therefore this amine was used in the next reaction



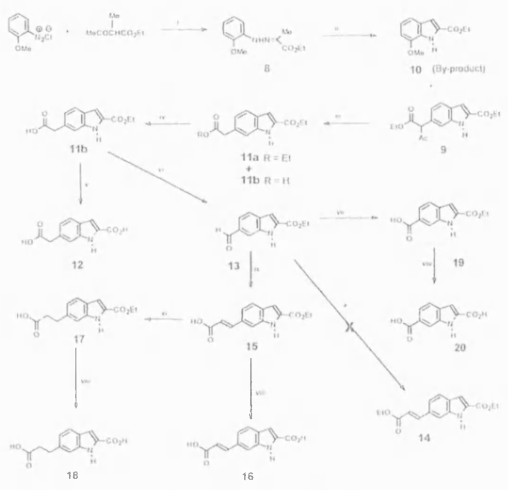
Scheme 1 *Reagents: i* $CHCl_3$, RT.; *ii* N-methyl-4-2-trichloroacetylpyrrole, $CHCl_3$

without further purification. 2-Carboxyindole-6-acetic acid (**12**) was treated with the amine (**3b**) using DCC as a coupling agent and DMAP as a catalyst. After purification by reverse phase HPLC the fractions containing the required material were freeze-dried immediately. The desired product **24** was obtained in a poor yield (13%) therefore an alternative coupling agent was sought. HBTU was generally found to be superior to DCC in this series, as shown by the following reactions. Coupling of the amine **3b** with the indole **20** gave rise to **25** in 52% yield, while coupling of the dicarboxylic acid **18**



Scheme 2 *Reagents: i* Pd–C, H2, DME, 40°C, *ii* 4-nitro-N-methylpyrrole-2carbonyl chloride, Et_3N, DME, *iii* EtOH (dry), HCl (g); *iv* NH_3 (dry), EtOH; Pd-C, H_2, EtOH, 60°C.

* To receive any correspondence.



Scheme 3 *Reagents: i* NaOAc, H_2O, EtOH, 5–7°C; *ii* $CH_3COCH_2CO_2Et$, TsOH, benzene; *iii* NaOH, EtOH, reflux; *iv* HCl in HOAc; *v* 1. KOH, EtOH, 2. HCl; *vi* pyridine *N*-oxide. Ac_2O; *vii* $KMnO_4$, actone, H_2O, 50°C; *viii* KOH, EtOH; *ix* pyridine, piperidine, malonic acid, 50°C; *x* (carbethoxymethyl)triphenylphosphonium bromide, *n*-BuLi; *xi* Pd-C, H_2.

produced **26** in 61% yield. Coupling of **16** gave **27** in 61% yield also. Compounds **24–27** were obtained as bis-TFA salts. Similar chemistry was used with the nitropyrrole **7a** (Scheme 2) to obtain **28**, after purification by HPLC, in 29% yield as a bis-TFA salt with no distinct melting point. In this case, the DCC route was superior.

As described elsewhere, several of these indole-containing netropsin analogues showed high affinity for AT rich regions of DNA. Compound **24** and compounds **26–28** all bound particularly strongly as shown by footprinting studies at 1 to 3 μM concentrations in the presence of similar concentrations of DNA. Compound **25**, however, was a poor ligand.[10] This result was not predicted by the design calculations[7,10] and might be explained by a different conformation that does not fit the double helix well.

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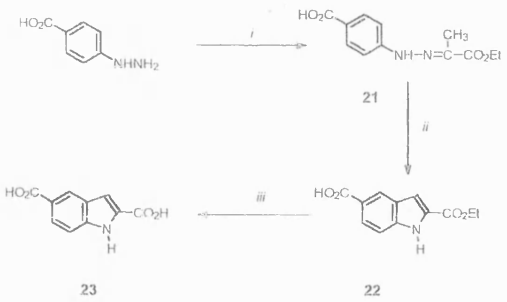

Scheme 4 Reagents: *i* CH_3COCO_2Et, HOAc, H2O; *ii* $ZnCl_2$ heat; *iii* 1. KOH, aq. EtOH; 2. HCl.

References

1. K.L. Kissinger, J.C. Dabrowiak and J.W. Lown. *Chem. Res. Toxicol.* 1990,**30**,162–168.
2. T.A. Beerman, J.M. Woynarowski, R.D. Sigmund, L.S. Gawron, K.E. Rao and J.W. Lown. *Biochim. Biophys. Acta* 1991,**1090**, 52–60.
3. W. Wang and J.W. Lown. *J. Med. Chem.* 1992, **35**, 2890–2897.
4. K.E. Rao, K. Krowicki, J. Balzarini, E. DeClercq, R.A. Newman, J.W. Lown. *Acta. Chim. Ther.* 1991, **18**, 21–42.
5. J.P. Bruzik, D.T. Auble and P.L. DeHaseth. *Biochemistry* 1987, **26**, 950–956.
6. M. McHugh, R.D. Sigmund and T.A. Beerman. *Biochem. Pharmacol* 1990, **39**, 707–714.
7. B. Waszkowycz. unpublished results.
8. K.E. Rao, J. Zimmermann and J.W. Lown. *J. Org.Chem.* 1991, **56**, 786–797.
9. R.V. Fishleigh, K.R. Fox, A.I. Khalaf, A.R. Pitt, M. Scobie, C.J. Suckling, J. Urwin, R.D. Waigh and S.C. Young. *J. Med. Chem.* (in press).
10. A.I. Khalaf, A.R. Pitt, M. Scobie, C.J. Suckling, R.D. Waigh, J. Urwin, R.V. Fishleigh, S.C.Young and K.R. Fox. *Tetrahedron* 2000, **56**, 5225–5239.
11. A.R. Kennedy, A.I. Khalaf, A.R. Pitt, M. Scobie, C.J. Suckling, J. Urwin and R.D. Waigh. *Acta Cryst. C* 1999, **C55**, IUC9900072.
12. E. Nishiwaki, S. Tanaka, H. Lee and M. Shibuya. *Heterocycles*, 1988, **27**, 1945–1952.
13. J.W. Lown and K. Krowicki. *J. Org. Chem.* 1985, **50**, 3774.
14. M. Julia and N. Prean-Joseph. *Compt. Rend. Acad. Sci*, 1963, **257**, 1115.
15. S. Penco, S. Redaelli and F. Arcamone. *Gazz. Chim. Ital.* 1967, **97**, 1110.

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Unexpected Dealkylation During Nucleophilic Substitution: Synthesis of 2-*N*,*N*-Dialkylamino Benzoxazoles and Benzothiazoles

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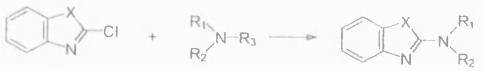
Abstract—Mild reaction conditions are described for the preparation of a number of 2-alkyl- and 2-arylaminobenzoxazoles and benzothiazoles from 2-chlorobenzoxazole and 2-chlorobenzothiazole and *N*-methyl or other simple *N*-alkyl tertiary amines. The reaction proceeds neat or in THF solution and involves dealkylation of the amine reactant by nucleophilic substitution by chloride. In the case of *N*-methylpyrrolidine and *N*-methylpiperidine demethylation was not observed and the major product was formed by ring opening to give chlorobutyl- and chloropentyl-methylamino substituted benzoxazoles and benzothiazoles. Treatment of the chlorobutyl derivative with iodide in acetone afforded the new 1*H*,2*H*,3*H*,4*H*,5*H*-[1,3]diazepino[2,1-*b*][1,3]benzoxazol-6-ium ring system.

As part of a programme of synthesis into minor groove binders for DNA,[1] we required to prepare distamycin analogues with heterocyclic head groups. Accordingly 2-chlorobenzoxazole was reacted with a distamycin analogue precursor that bore both a primary and a tertiary amine. Surprisingly, substitution to afford the corresponding aminobenzoxazole occurred at both amino groups with concomitant demethylation or de-ethylation of the tertiary amine.[2–6] This unexpected reaction of the tertiary amines prompted us to investigate the generality of the reaction. Although the products of these reactions, *N*,*N*-dialkylamino benzoxazoles and benzothiazoles, have been prepared before,[10–12] most recently using high pressure,[4] (see Table 1 for further references) the reactions described here offer simpler and more direct routes to these compounds.

Treatment of 2-chlorobenzoxazole (**1a**) with *N*-methylmorpholine (**2a**) under reflux in THF (or neat at 130°C) afforded 2-*N*-morpholinobenzoxazole (**3a**) in good yield (85%) after purification through a short column. In THF solution, 2-chlorobenzothiazole (**1b**) was much less reactive giving the corresponding morpholinothiazole (**3b**) in only 8% yield. However in the absence of solvent a significantly increased yield (50%) was obtained. Concentration and temperature therefore seem to be important in this new reaction.



1a X = O
1b X = S

2 **3**

The scope of this substitutive dealkylation reaction was studied to examine the influence of ring alkyl substitution in the tertiary amines. *N*,*N*-Dimethylbenzylamine underwent substitution with loss of the benzyl group to give **3d** (87%) but *N*,*N*-dimethylaniline afforded 2-*N*-methyl-*N*-phenylbenzoxazole (**3f**) in moderate yield (43%) with loss of the methyl group. Triethylamine reacted similarly to the other trialkylamines giving the diethylamino derivative **3e** in 89% yield. On the other hand, dicyclohexylmethylamine and *N*,*N*-diethylaniline failed to react. These results and others (Table 1) show that the normal effects of reactivity in nucleophilic substitution are followed. Attack of the nucleophile on the aryl halide is subject to steric hindrance and substitution is preferred at benzylic sites.

The reaction mechanism suggested by these observations (Scheme 1) involves initial addition of the tertiary amine to the heteroaryl chloride giving an adduct with a positively charged nitrogen atom. Instead of chloride acting as a leaving group, the adduct decomposes by nucleophilic substitution with the quaternary ammonium salt acting as a leaving group. Once chloride has been formed in the aprotic reaction mixture, it is a potential nucleophile leading to alkyl chlorides as the by-products. Benzyl chloride was indeed detected from the ^{1}H NMR signal at δ 4.6 in the

Keywords: 2-arylaminobenzoxazoles; 2-chlorobenzoxazole; 2-chlorobenzothiazole; dealkylation; [1,3]diazepino[2,1-b][1,3]benzazoles.
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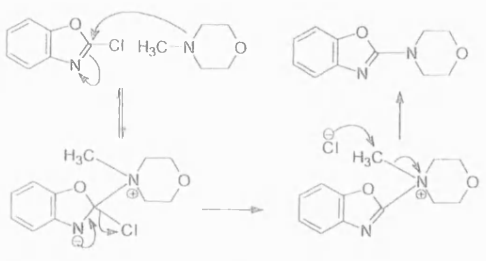
Table 1. Synthesis of **3** from 2-chlorobenzoxazole (or 2-chlorobenzothiazole) and tertiary amines

Entry	Product	X	R^1	R^2	R^3	Yield%	Mp (°C)
1	**3a**	O		Morpholino	CH_3	80	92–95[a]
2	**3b**	S		Morpholino	CH_3	50	122–125[b]
3	**3c**	O	CH_3	CH_3	CH_2Ph	87	89–91[c]
4	**3d**	S	CH_3	CH_3	CH_2Ph	72	84–86[d]
5	**3e**	O	CH_2CH_3	CH_2CH_3	CH_2CH_3	89	Oil[4,15]
6	**3f**	O	CH_3	Ph	CH_3	43	Oil[g]
7	**3g**	O		Pyrrolidino	CH_3	7[e]	134–135[f]
8	**3h**	S		Pyrrolidino	CH_3	7[e]	96–98[g]
9	**3i**	O		Piperidino	CH_2CH_3	86	72–73[h]
10	**3j**	S		Piperidino	CH_2CH_3	41	90–91[i]
11	**3k**	S	CH_3	Ph	CH_3	17	Oil[j]

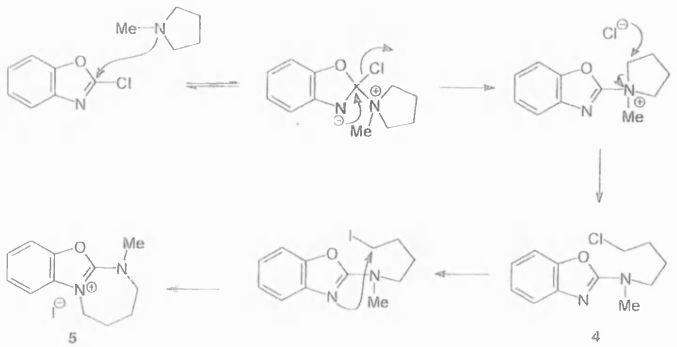
[a] Lit.[13] mp 88–89°C.
[b] Lit.[14] mp 126–127°C.
[c] Lit.[13] mp 88–89°C.
[d] Lit.[13] mp 84–86°C.
[e] Indicates that the major product was a ring opened derivative (see below).
[f] Lit.[9] mp 136–137°C.
[g] Lit.[16] mp 98–100°C.
[h] Lit.[17] mp 70–71°C.
[i] Lit.[7] mp 93–95°C.
[j] Lit.[18] mp 66–67°C.



Scheme 1. Proposed mechanism of dealkylative substitution.

reaction mixture with benzyldimethylamine as substrate and the increase of the alkyl chloride was followed during the course of the reaction. The action of chloride and bromide as nucleophiles in substitution reactions of ammonium salts, sulphonium salts, and oxonium salts is well known. It is also possible that attack by chloride on the methyl group is internal, i.e. the two stages are concerted, a reasonable suggestion because of the proximity of the departing chloride and the adjacent polarised C–N bond.

A further unusual observation was made. When the products of the reactions of 2-chlorobenzoxazole and 2-chlorobenzothiazole with *N*-methylpyrrolidine or *N*-ethylpyrrolidine were purified, a second product was isolated along with the expected adducts **3g** and **3h**. The new products contained covalently bound chlorine and the methyl group was still present; they were insoluble in water and clearly not salts. In the case of the compound derived from *N*-methylpyrrolidine, high resolution mass spectroscopy established the molecular formula $C_{14}H_{19}ClN_2O$. The ^{13}C NMR spectrum showed four distinct methylene groups deriving from the pyrrolidine ring suggesting that the ring opened structure **4** (Scheme 2) represented the new compound. This suggestion was also consistent with the long range couplings shown by COSY experiments: an interaction between the *N*-methyl protons (δ 3.20) and the methylene protons (δ 3.68) was observed. What is not easily understood is that this reaction is strongly favoured in the case of pyrrolidine. A small amount of the analogous product was characterised from the reaction of *N*-ethylpiperidine and 2-chlorobenzoxazole but in other cases no ring cleavage product was detected, although it may have been present in small quantity. Again, it is possible that



Scheme 2. Proposed mechanism of formation of ring opened products and further cyclisation.

release of chlorine from the oxazole ring and attack on the pyrrolidine carbon is a concerted process, which may be sterically favoured in the five-membered ring. To confirm the presence of the chloromethyl group, compound 4 was treated with sodium iodide in acetone at room temperature. This surprisingly led to the formation of a new fused heterocyclic system, 1-Methyl-1*H*,2*H*,3*H*,4*H*,5*H*-[1,3]diazepino[2,1-*b*][1,3]benzoxazol-6-ium iodide, **5**. Evidence for this structure comes from the water solubility of 5 and the downfield shift of the methylene protons (δ_H 3.68–4.02 and 4.48) and carbons (δ_C 44.48 and 49.55–47.14 and 54.48) and *N*-methyl protons (δ_H 3.20–3.50) and carbons (δ_C 35.42–41.97). Symmetrical structures such as pyrrolidinium salts are ruled out (Scheme 2).

Recently, further reactions under high pressure have been described for the preparation of 4-dialkyl-aminopyridines.[19] It would be interesting to discover whether milder conditions such as those described here would be successful in that case also.

Experimental

The majority of compounds prepared by the new route have been described before. Physical constants for compounds obtained and literature comparisons are given in Table 1. Spectroscopic data are reported below with the preparative details. IR spectra: solids were run as KBr discs and liquids as films, using a Nicolet Impact 400D. Low- and high-resolution (EI-MS) mass spectra were obtained on a Jeol JMS-AX505HA mass spectrometer. NMR spectra were obtained on a Bruker AMX 400 spectrometer operating at 400 MHz for ^{1}H and 100.6 MHz for ^{13}C. Column chromatography was performed with silica gel Prolabo (200–400 mesh).

2-*N*-morpholinobenzoxazole (3a).[13] *N*-Methylmorpholine (329 mg, 3.256 mmol) was dissolved in THF (20 mL, dry) to which 2-chlorobenzoxazole (504 mg, 3.256 mmol) was added at room temperature with stirring. The reaction mixture was heated under reflux for 3 h then the solvent removed under reduced pressure at 50°C. The residue was dissolved in a small amount of ethyl acetate and applied to a silica gel column. Ethyl acetate/*n*-hexane (1:1 v/v) was used to elute the product, which was obtained as a pale yellow solid (529 mg, 80%); δ_H ($CDCl_3$): 3.62–3.65 (4H, t, *J*=4.8 Hz, CH_2–N–CH_2); 3.83–3.86 (4H, t, *J*=4.8 Hz, CH_2–O–CH_2); 7.06 (1H, dt, *J*=1.2 and 7.8 Hz, Ar*H*); 7.19 (1H, dt, *J*=1.2 and 7.8 Hz, Ar*H*); 7.26 (1H, d, *J*=7.8 Hz, Ar*H*); 7.38 (1H, d, *J*=7.8 Hz, Ar*H*). ν_{max}: 1670, 1580, 1461, 1290, 1108, 763, 744 cm^{-1}.

2-*N*,*N*-dimethylaminobenzoxazole (3c).[13] *N*,*N*-Dimethylbenzylamine (507 mg, 3.750 mmol) and 2-chlorobenzoxazole (576 mg, 3.750 mmol) were heated at 130°C for 3 h. The reaction mixture was left to cool to room temperature then the crystalline mass was dissolved in hot ethyl acetate and applied to a silica gel column. The required benzoxazole (3c) was obtained as white crystalline material by elution with ethyl acetate/*n*-hexane (1:1 v/v) (527 mg, 87%). δ_H ($CDCl_3$): 3.21 (6H, s, NMe_2); 7.00 (1H, dt, *J*=1.2 and 7.7 Hz, Ar*H*); 7.16 (1H, dt, *J*=1.2 and 7.8 Hz, Ar*H*); 7.25 (1H, d, *J*=7.8 Hz, Ar*H*); 7.36 (1H, d, *J*=7.8 Hz, Ar*H*). δ_C ($CDCl_3$): 37.65, 108.55, 115.98, 120.16, 123.82, 143.58, 149.07, 163.05. ν_{max}: 1665, 1588, 1462, 1426, 901, 745 cm^{-1}.

Similarly the following compounds were prepared.

2-*N*-Morpholinobenzothiazole (3b).[14] As a pale yellow crystalline solid (50%); δ_H ($CDCl_3$): 3.64 (4H, t, *J*=4.8 Hz, CH_2NCH_2); 3.85 (4H, t, *J*=4.7 Hz, CH_2OCH_2); 7.11 (1H, dt, *J*=1.2 and 7.4 Hz, Ar*H*); 7.32 (1H, dt, *J*=1.2 and 7.4 Hz, Ar*H*); 7.58 (1H, d, *J*=7.4 Hz, Ar*H*); 7.62 (1H, d, *J*=7.4 Hz, Ar*H*). ν_{max}: 1596, 1562, 1539, 1443, 1291, 1116, 759, 727 cm^{-1}. HREIMS: found 220.066. Calculated for $C_{11}H_{12}N_2OS$ 220.067.

2-*N*,*N*-Dimethylaminobenzothiazole (3d).[13] As a white crystalline solid (72%). δ_H ($CDCl_3$): 3.21 (6H, s, NMe_2); 7.06 (1H, dt, *J*=1.2 and 7.4 Hz, Ar*H*); 7.29 (1H, dt, *J*=1.2 and 7.4 Hz, Ar*H*); 7.57 (1H, d *J*=7.4 Hz, Ar*H*); 7.60 (1H, d, *J*=7.4 Hz, Ar*H*). δ_C ($CDCl_3$): 40.40, 118.99, 120.81, 121.10, 126.13, 131.34, 153.47, 168.99. ν_{max}: 1599, 1574, 1546, 1453, 1416, 1295, 750, cm^{-1}.

2-*N*,*N*-Diethylaminobenzoxazole (3e).[4,15] As a colourless oil (89%). δ_H ($CDCl_3$): 1.27–1.31 (6H, t, *J*=7.1 Hz, NCH_2CH_3); 3.57–3.62 (4H, q, *J*=7.1 Hz, NCH_2CH_3); 6.96–7.01 (1H, dt, *J*=1.1 and 7.8 Hz, Ar*H*); 7.12–7.16 (1H, dt, *J*=1.1 and 7.8 Hz, Ar*H*); 7.25 (1H, d, *J*=7.8 Hz, Ar*H*); 7.36 (1H, d, *J*=7.8 Hz, Ar*H*). δ_C ($CDCl_3$): 13.44, 42.91, 108.45, 115.77, 119.89, 123.73, 143.65, 148.81, 162.20. ν_{max}: 2968, 2937, 1647, 1580, 1466, 1399, 1251, 784, 761, 748 cm^{-1}.

2-*N*-Methyl-*N*-phenylaminobenzoxazole (3f).[8] As a colourless oil (43%). δ_H ($CDCl_3$): 3.64 (3H, s, N*Me*); 7.03–7.08 (1H, dt, *J*=1.1 and 7.8 Hz, Ar*H*); 7.18–7.22 (1H, dt, *J*=1.1 and 7.7 Hz, Ar*H*); 7.25–7.31 (2H, m, Ar*H*); 7.42–7.48 (5H, m, Ar*H*). δ_C ($CDCl_3$): 39.04, 108.94, 116.66, 121.07, 123.99, 124.43, 126.02, 129.24, 142.76, 142.95, 148.74, 161.26. ν_{max}: 3063, 2940, 1632, 1570, 1503, 1380, 1246, 1138, 755, 747, 701 cm^{-1}.

Reaction of *N*-methylpyrrolidine and (1a): formation of *N*-(4-chlorobutyl)-*N*-methylaminobenzoxazole. 2-Chlorobenzoxazole (460 mg, 3.0 mmol) and *N*-methylpyrrolidine (260 mg, 3.0 mmol) were dissolved in THF (20 mL, dry). The reaction mixture was heated under reflux for 6 h. The solvent was removed under reduced pressure and the residue purified by column chromatography on silica eluted with ethyl acetate/*n*-hexane (1:20 v/v). The first fraction (compound of type **4**, *N*-(4-chlorobutyl)-*N*-methylaminobenzoxazole) was obtained as a colourless oil (617 mg, 86%). δ_H ($CDCl_3$): 1.85 (4H, m, $(CH_2)_2CH_2N$); 3.20 (3H, s, N*Me*); 3.68 (4H, m, CH_2Cl and CH_2N); 7.01 (1H,dt, *J*=1.2 and 7.8 Hz, Ar*H*); 7.15 (1H, dt, *J*=1.2 and 7.8 Hz, Ar*H*); 7.26 (1H, d, *J*=7.8 Hz, Ar*H*); 7.36 (1H, d, *J*=7.8 Hz, Ar*H*). δ_C ($CDCl_3$): 24.78, 29.48, 35.42, 44.48, 49.55, 108.55, 115.96, 120.18, 123.85, 143.45, 148.85, 162.65. ν_{max}: 2956, 2867, 1598, 1567, 1544, 1447, 1293, 753, 727 cm^{-1}. HREIMS: found 238.087, calculated for $C_{12}H_{15}N_2O^{35}Cl$ 238.087, and found 240.086 calculated for $C_{12}H_{15}N_2O^{37}Cl$ 240.086.

The second fraction was obtained as white solid material (40 mg, 7%), identified as **3g**.[9] δ_H ($CDCl_3$): 2.05 (4H, t, J=8 Hz, $(CH_2)_2CH_2N$); 3.66 (4H, t, J=4.0 Hz, CH_2N); 6.99 (1H, t, J=7.8 Hz, Ar*H*); 7.15 (1H, t, J=7.8 Hz, Ar*H*); 7.26 (1H, d, J=7.8 Hz, Ar*H*); 7.36 (1H, d, J=7.8 Hz, Ar*H*). δ_C ($CDCl_3$): 25.58, 47.40, 108.55, 115.95, 120.04, 123.81, 143.58, 149.00, 160.98. ν_{max}: 2922, 1642, 1584, 800, 760, 740 cm^{-1}.

Reaction of *N*-ethylpiperidine and (1a): *N*-(4-chloropentyl)-*N*-ethylaminobenzoxazole. 2-Chlorobenzoxazole (460 mg, 3.0 mmol) and 1-ethylpiperidine (679 mg, 6.0 mmol) were dissolved in THF (20 mL, dry). The reaction mixture was heated under reflux for 24 h. The solvent was removed under reduced pressure and the residue purified by column chromatography eluted with ethyl acetate/*n*-hexane (1:9 v/v). The first fraction was obtained as a white solid which was identified as **3i**,[17] (519 mg, 86%). δ_H ($CDCl_3$): 1.73 (6H, m, 3×CH_2); 3.71 (4H, m, CH_2N); 7.04 (1H, dt, J=1.2 and 7.8 Hz, Ar*H*); 7.19 (1H, dt, J=1.2 and 7.8 Hz, Ar*H*); 7.28 (1H, d, J=7.8 Hz, Ar*H*); 7.39 (1H, d, J=7.8 Hz, Ar*H*). δ_C ($CDCl_3$): 24.08, 25.24 (2×C); 46.62 (2×C); 108.54, 116.00, 120.26, 123.82, 143.39, 148.71, 162.47. ν_{max}: 2941, 2854, 1643, 1576, 1454, 1275, 792, 754, 743 cm^{-1}. A second fraction (compound of type 4, *N*-(4-chloropentyl)-*N*-ethylaminobenzoxazole) was obtained as a colourless oil (55 mg, 7%). δ_H ($CDCl_3$): 1.27–1.31 (3H, t, J=7.1 Hz, CH_2CH_3), 1.49–1.58 (2H, m, CH_2), 1.69–1.77 (2H, m, CH_2), 1.82–1.89 (2H, qt, J=6.7 Hz, CH_2), 3.51 (2H, t, J=7.4 Hz, CH_2N), 3.53 (2H, t, J=6.7 Hz, CH_2Cl), 3.57 (2H, q, J=7.3 Hz, NCH_2CH_3), 6.99 (1H, dt, J=1.2 and 7.8 Hz, Ar*H*), 7.15 (1H, dt, J=1.2 and 7.8 Hz, Ar*H*), 7.27 (1H, d, J=7.8 Hz, Ar*H*), 7.34 (1H, d, J=7.8 Hz, Ar*H*). δ_C ($CDCl_3$): 13.29, 24.09, 27.54, 32.30, 43.47, 44.80, 48.01, 108.53, 115.89, 120.04, 123.83, 148.82, 162.47. ν_{max}: 2939, 1649, 1585, 1462, 760, 745 cm^{-1}. HREIMS: found 266.119, calculated for $C_{14}H_{19}N_2O^{35}Cl$ 266.119; and found 268.116, calculated for $C_{14}H_{19}N_2O^{37}Cl$ 268.116.

Reaction of (1b) with *N*-methylpyrrolidine: formation of *N*-(4-chlorobutyl)-*N*-methylaminobenzothiazole. The same experimental procedure as above was employed to give two products eluted with ethyl acetate/petroleum ether (1:3 v/v). The first fraction (compound of type 4, *N*-(4-chlorobutyl)-*N*-methylaminobenzothiazole) was obtained as a colourless oil (83%). δ_H ($CDCl_3$): 1.82–1.85 (4H, m, 2×CH_2), 3.18 (3H, s, N*Me*), 3.56–3.59 (4H, m, NCH_2 and CH_2Cl), 7.06 (1H, dt, J=1.2 and 7.8 Hz, Ar*H*), 7.29 (1H, dt, J=1.2 and 7.8 Hz, Ar*H*), 7.55 (1H, d, J=7.8 Hz, Ar*H*), 7.59 (1H, d, J=7.8 Hz, Ar*H*). δ_C ($CDCl_3$): 24.84, 29.84, 38.14, 44.78, 52.47, 118.97, 120.77, 121.14, 126.13, 131.04, 153.37, 168.50. ν_{max}: 2956, 2867, 1598, 1544, 1447, 1293, 754, 727 cm^{-1}. HREIMS: found 254.065 calculated for $C_{12}H_{15}N_2S^{35}Cl$ 254.064; and found 256.063 calculated for $C_{12}H_{15}N_2S^{37}Cl$ 256.063. The second fraction (**3h**) was obtained as a white solid (7%).[16] δ_H ($CDCl_3$): 2.05 (4H, t, J=8.0 Hz, 2×CH_2); 3.66 (4H, t, J=4.0 Hz, 2×NCH_2); 7.03–7.06 (1H, dt, J=1.1 and 7.8 Hz, Ar*H*); 7.27–7.31 (1H, dt, J=1.1 and 7.8 Hz, Ar*H*); 7.57–7.61 (2H, m, Ar*H*). δ_C ($CDCl_3$): 25.88, 49.71, 118.91, 120.88, 126.12, 131.96, 153.55, 165.58. ν_{max}: 2922, 1642, 1583, 1459, 761 cm^{-1}.

2-Piperidinobenzothiazole (3j). 2-Chlorobenzothiazole (504 mg, 2.971 mmol) and *N*-ethylpiperidine (1.009 g, 8.913 mmol) were heated at 130°C for five days. Excess reagent was removed under reduced pressure and the crude product was applied to a column chromatography. Ethyl acetate/*n*-hexane 1/10 was used to elute the product which was obtained as pale yellow crystalline material (**3j**) (266 mg, 41% yield), R_f=0.33. δ_H ($CDCl_3$): 1.68 (6H, br s, 3×CH_2); 3.56 (4H, br s, CH_2NCH_2); 7.03–7.07 (1H, dt, J=1.1 and 7.8 Hz, Ar*H*); 7.26–7.31 (1H, dt, J=1.1 and 7.8 Hz, Ar*H*); 7.55–7.59 (2H, m, Ar*H*). δ_C ($CDCl_3$): 24.67, 25.71 (2×C), 50.02 (2×C), 119.21, 120.97, 121.44, 126.26, 131.12, 153.42, 169.25. ν_{max}: 2945, 2924, 2846, 1593, 1561, 1535, 1444, 1261, 762, 732 cm^{-1}.

2-*N*-methyl-*N*-phenylaminobenzothiazole (3k).[18] The same procedure for the synthesis of (**3f**) was employed to give the product as a colourless oil. δ_H ($CDCl_3$): 3.66 (3H, s, N*Me*); 7.09 (1H, dt, J=1.1 and 7.8 Hz, Ar*H*); 7.30–7.52 (7H, m, Ar*H*); 7.66 (1H, d, J=7.8 Hz, Ar*H*). δ_C ($CDCl_3$): 40.59, 119.36, 120.60, 121.88, 126.00, 126.12 (2×C), 127.55, 130.06 (2×C), 131.33, 145.96, 152.78, 168.37. ν_{max}: 3063, 1593, 1521, 755 cm^{-1}.

1-Methyl-1*H*,2*H*,3*H*,4*H*,5*H*-[1,3]diazepino[2,1-*b*][1,3]benzoxazol-6-ium iodide (compound of type 5). *N*-(4-chlorobutyl)-*N*-methylaminobenzoxazole (20 mg, 0.084 mmol) was dissolved in dry acetone (5 mL) to which was added anhydrous sodium iodide (25 mg, 0.10 mmol). The solution was heated under reflux for 8 h and allowed to cool to room temperature. The sodium chloride precipitate was filtered and the solvent evaporated to dryness under reduced pressure, to afford the product as a white solid (25 mg, 90%). mp>240°C. δ_H ($CDCl_3$): 2.27–2.39 (4H, m, 2×CH_2), 3.50 (3H, s, N*Me*), 4.02 (2H, t, J=5.6 Hz, NCH_2), 4.48 (2H, t, J=5.6 Hz, N^+CH_2), 7.31–7.36 (1H, m, Ar*H*), 7.39–7.47 (3H, m, Ar*H*). δ_C ($CDCl_3$): 24.60, 24.42 (CH_2–CH_2), 41.97 (N*Me*), 47.14 (NCH_2), 54.48 (N^+CH_2), 111.46, 111.91, 125.54, 126.67 (CH of Ar), 131.92, 144.35 (C of Ar), 158.6 (N^+=*C*–O(N)). HRFABMS Found 203.11834 (95%, base peak), calculated for $C_{12}H_{15}N_2O$ 203.11844 (M^+-I^-). ν_{max} 1685, 1480, 1400, 1267, 1164, 758 cm^{-1}.

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References

1. (a) Khalaf, A. I.; Pitt, A. R.; Scobie, M.; Suckling, C. J.; Urwin, J.; Waigh, R. D.; Young, S. C.; Fishleigh, R. V.; Fox, K. R. *J. Med. Chem.* in press. (b) Khalaf, A. I.; Pitt, A. R.; Scobie, M.; Suckling, C. J.; Urwin, J.; Waigh, R. D.; Young, S. C.; Fishleigh, R. V. *Tetrahedron* **2000**, *56*, 5225–5239. (c) Khalaf, A. I.; Pitt, A. R.; Scobie, M.; Suckling, C. J.; Urwin, J.; Waigh, R. D.; Young, S. C.; Fishleigh, R. V. *J. Chem. Res., Synopsis* **2000**, 246–265; *J. Chem. Res., Miniprint* 0751–0770.

2. Brown, H. C. *J. Am. Chem. Soc.* **1945**, *67*, 378–380.

3. (a) Miller, J. *Aromatic Nucleophilic Substitution*; Elsevier: Amsterdam, 1968. (b) Bacaloglu, R.; Blasko, A.; Bunton, C. A.;

Dorwin, E.; Ortega, F.; Zucco, C. *J. Am. Chem. Soc.* **1991**, *113*, 238–246.
4. Matsumoto, K.; Hashimoto, S.; Otani, S. *J. Chem. Soc., Chem. Commun.* **1991**, 306–307.
5. Kukhar, V. P. *J. Org. Chem. USSR (Engl. Transl.)* **1978**, *14*, 1710–1714.
6. Hervens, F.; Viehe, H. G. *Angew. Chem., Int. Ed. Engl.* **1973**, *12* (5), 405–406 (see also 1991).
7. Nagarajan, K.; Kulkarni, C. L.; Shah, R. K. *Indian J. Chem.* **1971**, *9*, 748–754.
8. Matsumoto, K.; Toda, M.; Hashimoto, S. *Chem. Lett.* **1991**, *8*, 1283–1286.
9. Hetzheim, A.; Schlaak, G.; Kerstan, C. *Pharmazie* **1987**, *42* (2), 80–82.
10. Elderfield, R. C.; Green, M. *J. Org. Chem.* **1952**, *17*, 431–441.
11. Hunziker, F.; Schindler, O. *Helv. Chim. Acta* **1965**, *48*, 1590–1597.
12. Davidkov, K.; Simov, D. *Dokl. Bolg. Akag. Nauk.* **1968**, *21* (11), 1193–1196 (*Chem. Abstr.* 70:57714k).
13. Benassi, R.; Grandi, R.; Pagnoni, U. M.; Taddei, F. *J. Chem. Soc., Perkin Trans. 2* **1985**, 1513–1522.
14. Da'mico, J. J.; Webster, S. T.; Campbell, R. H.; Twine, C. E. *J. Org. Chem.* **1965**, *30*, 3618–3625.
15. Advani, S. P.; Sam, J. *J. Pharm. Sci.* **1968**, *57*, 1693–1696.
16. Claude, S.; Tabacchi, R.; Duc, L.; Fuchs, R.; Boosen, K. *Helv. Chim. Acta* **1980**, *63* (3), 682–692.
17. Eilingsfeld, H.; Moebius, L. *Chem. Ber.* **1965**, *98*, 1293–1307.
18. D'Amico, J. J.; Tung, C. C.; Walker, L. A. *J. Am. Chem. Soc.* **1959**, *81*, 5957–5959.
19. Kotsuki, H.; Sakai, H.; Shinohara, T. *Synlett* **2000**, 116–118.

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A Method for the Determination of Substitution Pattern in Coumarins using a Lanthanide Shift Reagent

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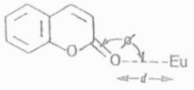
Summary The n.m.r. shift reagent tris-(7,7-dimethyl-1,1,1,2,2,3,3-heptafluoro-octane-4,6-dionato)europium(III) [Eu(fod)$_3$] complexes with the carbonyl oxygen of coumarins so that all shifts can be related to a position for the europium atom in the plane of the ring and on the 3-H side of the C=O bond; relatively large shifts are produced for an 8-substituent, and information about other substituent positions can be obtained.

MANY coumarins have been isolated from plants and although modern techniques allow rapid identification of attached groups, the substitution pattern on the benzene ring may be difficult to determine. Others have attempted to correlate the substitution pattern empirically with such data as u.v. absorption[1] and chemical shift,[1,2] and non-empirically, with long-range coupling constants[2] and internal nuclear Overhauser effects.[3] The latter is certainly the most powerful technique but is technically more difficult and less accessible.



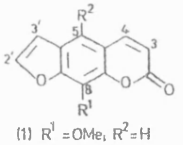
FIGURE

Normally the use of a lanthanide shift reagent to differentiate isomers requires the three-dimensional location of the lanthanide atom in the complex, a process needing a computer to achieve a 'best fit' with the observed shifts.[4] In the present work we have striven for a simpler practical approach by reducing the problem to two dimensions, regarding the coumarin rings as planar and assuming coplanarity with the europium atom. In this way it is easy to fix the bond angle (ϕ, Figure) and O–Eu distance (d, Figure) using a field map[5] which effectively gives lines of equal induced shift around the lanthanide atom. The coumarin 3-H is shifted most, and the shifts of the other protons are most conveniently expressed relative to shift (3H) = 1. With these ratios measured, a stereomodel of the coumarin may be placed on the (suitably scaled) field map, to give values of ϕ and d which can be used to measure θ and R for substitution in the McConnell–Robertson equation[4]

$$\Delta\nu\,(\mathrm{Eu}) = \frac{3\cos^2\theta - 1}{R^3} \times \text{constant}$$

where θ is the O–Eu–H bond angle and R the H–Eu distance.

The constant can be ignored since it is the same for all protons within the molecule. Calculation of values of $(3\cos^2\theta - 1)/R^3$ for each proton then gives ratios of shifts which in many cases will fit only one likely structure. In all cases examined so far $\phi = 150 \pm 10°$ and $d = 3 \pm 0{\cdot}5$ Å.



(1) R^1 = OMe, R^2 = H
(2) R^1 = H, R^2 = OMe

We have taken as an example the results for two isomeric methoxyfuranocoumarins, xanthotoxin (1) and bergapten (2), whose structures have been determined by synthesis.[6] The observed and calculated shifts are given in the Table, with the values for ϕ and d obtained by trial. From u.v. spectra both must be linear. The calculated shifts for the methoxys were obtained in the maximally out-of-plane conformations, which correspond to minimum steric interactions in the stereomodels.

TABLE

			Shifts/p.p.m.: observed (calculated)						
Compound	ϕ	d (Å)	3-H	4-H	5-H	8-H	2′-H	3′-H	OMe
(1)	145	2·75	1(1)	0·25(0·26)	0·20(0·18)	—	0·10(0·10)	0·11(0·10)	0·29(0·29)
(2)	158	3·45	1(1)	0·34(0·33)	—	0·30(0·32)	0·11(0·09)	0·12(0·11)	0·14(0·13)

The O–Me shift in xanthotoxin is so large that the methoxy can only be in the 8-position of the coumarin and in bergapten the shifts clearly demonstrate that the 8-position is unsubstituted.

In view of the good correlations contact shifts can be ignored, as can complexation with ether oxygens, but obviously in compounds with more powerful competing complexation sites the simple approach can only be maintained by masking the alternative electron donors. We have found, for example, that formation of the trimethylsilyl ether is an effective way of preventing interaction with the side-chain –OH groups commonly encountered in naturally occurring coumarins.

Our confidence in the generality of our method is based on experiments using Eu(fod)$_3$ with 6-methylcoumarin, herniarin,[7] 4-methylherniarin, imperatorin[7] and xanthoxyletin.[7] Several additions of Eu(fod)$_3$ were used for each compound, and the relative shifts obtained graphically where possible.

We have used the method as an aid[8] in resolving the final uncertainties concerning the structure of avicennin.[9]

Variations in ϕ and d make strict comparison between compounds difficult, but the shifts for similar substituents are always $3 >> 4 \simeq 8 > 5 \simeq 7 > 6$.

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[1] B. E. Nielsen, *Dansk Tidsskr. Farm.*, 1970, **44**, 111.
[2] W. Steck and M. Mazurek, *Lloydia*, 1972, **35**, 418.
[3] T. Tomimatsu, M. Hashimoto, T. Shiugu, and K. Tori, *Chem. Comm.*, 1969, 168.
[4] The whole field of lanthanide shift reagents has recently been reviewed: A. F. Cockerill, G. C. O. Davies, R. C. Harden, and D. M. Rackham, *Chem. Rev.*, 1973, **73**, (6), 553.
[5] R. M. Wing, T. A. Early, and J. J. Uebel, *Tetrahedron Letters*, 1972, 4153.
[6] F. M. Dean, 'Naturally Occurring Oxygen Ring Compounds,' Butterworths, London, 1963, pp. 198—208.
[7] For structures and other data see ref. 1.
[8] A. I. Gray, R. D. Waigh, and P. G. Waterman, in preparation.
[9] H. R. Arthur and W. D. Ollis, *J. Chem. Soc.*, 1963, 3910.

Avicennol: a New Pyranocoumarin from the Root-bark of *Zanthoxylum avicennae*, and its Conversion into Avicennin

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A new angular pyranocoumarin, avicennol (4) {6-(3-hydroxy-3-methyl-*trans*-but-1-enyl)-5-methoxy-2,2-dimethyl-2*H*-benzo[1,2-*b*:3,4-*b*']dipyran-8-one}, has been isolated from the root-bark of *Zanthoxylum avicennae* (Rutaceae): the structural assignment is based on chemical and spectral evidence. A lanthanide shift reagent and the internal nuclear Overhauser effect have been used to differentiate between possible isomers. Avicennol has been converted into avicennin, previously reported from this and other species; thus the total structure of avicennin is determined. From this species avicennin may be an artefact.

PREVIOUS examinations of the root-bark of *Zanthoxylum avicennae* (Lam.) DC (family Rutaceae; sub-family Rutoideae) yielded the flavonoid glycosides hesperidin and diosmin,[1a] the benzophenanthridine alkaloid avicine,[1b] and a novel pyranocoumarin, avicennin.[1c] The structure of avicennin was partially defined by Arthur and Ollis,[2] leaving three possible isomers (1)—(3). Avicennin has also been isolated from a second rutaceous species, *Eriostemon coccineus* C. A. Gard.[3] We now report the isolation and structural elucidation of a novel coumarin, avicennol (4), from the root-bark of *Zanthoxylum avicennae* and confirm the structure of avicennin as (2) by its synthesis from avicennol.

The light petroleum extract of the ground root-bark gave, on cooling, a high yield (2·3%) of yellow crystalline material which proved to be a single compound (t.l.c.). U.v. and i.r. spectra indicated that the compound possessed a coumarin nucleus,[4] probably having extended conjugation and with a non-phenolic hydroxy-group. Elemental analysis and accurate mass measurement gave the molecular formula $C_{20}H_{22}O_5$. The mass fragment-

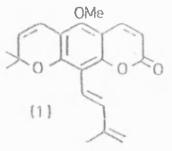
[1] (*a*) H. R. Arthur, W. L. Hui, and C. N. Ma, *J. Chem. Soc.*, 1956, 632; (*b*) H. R. Arthur, W. H. Hui, and Y. L. Ng, *ibid.*, 1959, 4007; (*c*) H. R. Arthur and C. M. Lee, *ibid.*, 1960, 4664.

[2] H. R. Arthur and W. D. Ollis, *J. Chem. Soc.*, 1963, 3910.

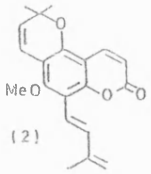
[3] P. W. Chow, A. M. Duffield, and P. R. Jeffries, *Austral. J. Chem.*, 1966, **19**, 483.

[4] B. E. Nielsen, *Dansk Tidsskr. Farm.*, 1970, **44**, 111.

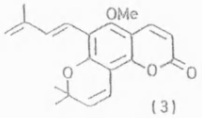
ation pattern was simple and suggested only loss of water and a methyl radical to give, as base peak, the ion $C_{10}H_{17}O_4$.



(1)



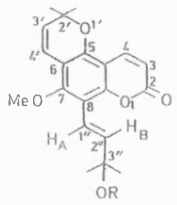
(2)



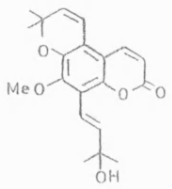
(3)

The n.m.r. spectrum defined all twenty-two protons. The occurrence of an AB quartet (J 10 Hz) with one half downfield at δ 8·06 confirmed the presence of the αβ-unsaturated δ-lactone ring of the coumarin nucleus.[4,5] A second AB quartet (J 10 Hz) and a signal for *gem*-methyl groups at δ 1·48 indicated the presence of the 2,2-dimethylchromen ring system encountered in pyranocoumarins.[6] A three-proton singlet at δ 3·80 indicated the presence of a methoxy-substituent, leaving only hydroxy- and *trans*-olefinic (J 16 Hz) protons and two further *gem*-methyl groups unaccounted for. As one of the two remaining positions on the benzenoid ring of the pyranocoumarin must be filled by the methoxy-group these signals can only be attributed to a single unit C_5H_9O. This can be accounted for by the unusual 3-hydroxy-3-methyl-*trans*-but-1-enyl side-chain previously encountered only by Chan *et al.*[7] in chromones from the rutaceous plant *Spathelia sorbifolia* L. Despite the helpful n.m.r. spectrum of avicennol there remained twelve possible isomers varying in the positioning of the substituents on the benzene ring.

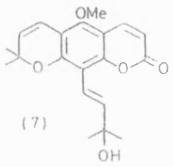
A large contribution to the determination of the absolute structure of avicennol was made by using the lanthanide shift reagent tris-(7,7-dimethyl-1,1,1,2,2,3,3-heptafluoro-octane-4,6-dionato)europium(III) [Eu(fod)$_3$] according to a method described elsewhere.[8] We have shown [8] that Eu(fod)$_3$ complexes with the carbonyl oxygen atom of coumarins in such a way that an 8-substituent shows a large downfield shift. With avicennol itself results were complicated by preferred complexation at the side-chain hydroxy-group, so this group was masked by formation of the *O*-trimethylsilyl derivative (5). Complexation then proceeded as expected. The same approach as previously [8] was used, and the best fit was obtained between observed and calculated shifts for values of ϕ (the C–O–Eu angle) = 152·5° and d (the O–Eu distance) = 2·75 Å. This gave exactly the observed shift for the H-4 (0·31), relative to H-3 * (1·00, by definition [8]) and reasonable values for all other shifts. We give in detail only the observed shifts for the side-chain olefinic protons



(4) R = H
(5) R = $SiMe_3$



(6)



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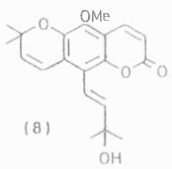
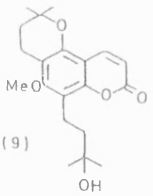


(8)



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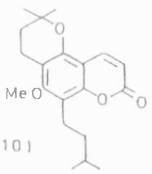

(10)

and those calculated with the side-chain in the four possible positions on the coumarin benzene ring (Table 1).

TABLE 1

Observed shifts for H_A and H_B of *O*-trimethylsilylavicennol (5) and their calculated values for attachment of the side-chain at positions 5, 6, 7, and 8 (relative of H-3 shift = 1·00)

		Calculated for			
	Observed	C-5	C-6	C-7	C-8
H_A	0·40	0·12	0·11	0·15	0·33
H_B	0·52	0·15	0·11	0·15	0·45

The calculations are based on average values for the two possible conformations of the side-chain double bond in

* In order to clarify the text, the non-systematic numbering system illustrated in formula (4) is used to refer to positions on the avicennol skeleton.

[5] E. V. Lassak and J. T. Pinhey, *J. Chem. Soc.* (*C*), 1967, 2000.
[6] T. Tomimatsu, M. Hashimoto, T. Shingu, and K. Tori, *Chem. Comm.*, 1969, 168.
[7] W. R. Chan, D. R. Taylor, and C. R. Willis, *J. Chem. Soc.* (*C*), 1967, 2540.
[8] A. I. Gray, R. D. Waigh, and P. G. Waterman, *J.C.S. Chem. Comm.*, 1974, 632.

the plane of the benzene ring and therefore are only approximate, since the side-chain conformation is not known. However, it is clear that only a side-chain in the 8-position offers anything approaching a shift of sufficient magnitude. This observation reduces the possible structures for avicennol from twelve to four (4) and (6)—(8). Further distinction between structures on the basis of $Eu(fod)_3$-induced shifts is less certain, since the shifts for protons of substituents in the 5-, 6-, and 7-positions are smaller, and so, therefore, are the calculated differences amongst the four remaining isomers. However, the observed methoxy-shift (0·15) offers clear evidence in favour of structures (4) and (6) (calculated shift 0·15) rather than (7) and (8) (calculated shift 0·11).

The structure (4) for avicennol was confirmed by measurement of the intramolecular nuclear Overhauser effect (n.O.e.) involving the methoxy-group and the olefinic protons; this technique was used earlier [6] for a similar pyranocoumarin. The observed n.O.e. is shown in Table 2. Structure (4) is the only one in which the

TABLE 2

Observed nuclear Overhauser effects for avicennol on saturation of the methoxy signal at δ 3·80

Increase in integrated intensities of signals of				
H-3	H-4	H-3′	H-4′	H-1″ and -2″
Nil	Nil	Nil	9%	5%

methoxy-group is close to both H-4′ and the side-chain olefinic protons, if structures which do not fit the shift reagent data are excluded.

It seemed possible that previous isolation [1c,2] of avicennin (in small quantity) rather than avicennol (in bulk) from *Z. avicennae* may have been due to differences in extraction and purification techniques, which in the earlier work involved treatment with acid, base, and boiling chloroform. Treatment of pure avicennol with acid and base as described [1c] gave no useful result, but phosgene quickly effected the dehydration, producing avicennin in high yield, with the same physical constants as already reported.[1c,2] Thus avicennin has the structure (2).

Catalytic hydrogenation of avicennol afforded two products: the expected tetrahydroavicennol (9), m.p. 165—165·5°, and hexahydroavicennin (10), m.p. 81—82°, for which the data obtained were in agreement with those reported by Arthur and Ollis.[2]

N.m.r. shift experiments with avicennin and hexahydroavicennin gave results substantially similar to those with the trimethylsilyl ether of avicennol.

EXPERIMENTAL

M.p.s were determined with a Kofler hot-stage apparatus. U.v. spectra (solvent ethanol) were recorded with a Unicam SP 800A spectrophotometer, and i.r. spectra (for KCl discs) with a Perkin-Elmer 237 grating spectrophotometer. Optical rotations were measured with a Bellingham and Stanley Polarimeter. N.m.r. spectra were recorded for solutions in deuterochloroform with a Perkin-Elmer R12 60 MHz instrument, with the exception of the n.O.e. experiment in which a Perkin-Elmer 90 MHz instrument was used. Mass spectra and accurate mass determinations were obtained with an A.E.I. MS 902 spectrometer.

Extraction of the Root-bark.—The dried, powdered root-bark (150 g) of *Zanthoxylum avicennae** was extracted (Soxhlet) with light petroleum (b.p. 40—60°; 3 l) for 10 h. On cooling, pale yellow crystals (3·55 g) were deposited from the extract. T.l.c. on alumina (Woelm; activity I), with chloroform–methanol (98 : 2) as eluant, showed a single spot (R_F 0·4) fluorescing orange under screened u.v. light (366 nm).

Avicennol (4).—Recrystallised from n-hexane–ethyl acetate (99 : 1) *avicennol* gave yellow plates, m.p. 124·5—125·5°, $[\alpha]_D^{21}$ 0° (*c* 1·00 in $CHCl_3$) (Found: C, 70·35; H, 6·65%; M^+, 342·1463. $C_{20}H_{22}O_5$ requires C, 70·2; H, 6·45%; *M*, 342·1467), $\lambda_{max.}$ 250 (log ε 4·51), 257 (4·61), and 301 nm (4·28), $\nu_{max.}$ 3480 (OH), 2960, 1725 (CO), 1585, 1255, and 1140 cm^{-1}, δ 1·48 (12H, s, 2′-Me_2, 3″-Me_2), 2·58 (1H, s, exchanged with D_2O, OH), 3·80 (3H, s, OMe), 6·65 and 5·69 (2H, ABq, *J* 10 Hz, H-4′ and -3′), 6·95 and 6·81 (2H, ABq, *J* 16 Hz, H-1″ and -2″), and 6·27 and 8·06 (2H, ABq, *J* 10 Hz, H-3 and -4), *m/e* 342 (M^+, 3%), 327 ($C_{20}H_{30}O_4$, 78), 309 ($C_{19}H_{17}O_4$, 100), 277 (14), 263 (6), 253 (5), 251 (8), and 162 (3).

3″-O-*Trimethylsilylavicennol* (5).—Avicennol (120 mg) was dissolved in benzene (sodium-dried) and an excess of *NO*-bis(trimethylsilyl)acetamide was added. The reaction was followed by t.l.c. on alumina with the system already described. After 4 h, the mixture was evaporated to dryness under reduced pressure, and the resulting yellow *solid* recrystallised from methanol; m.p. 100—101° (Found: M^+, 414·1840. $C_{23}H_{30}O_5Si$ requires *M*, 414·1862), $\lambda_{max.}$ 251 (log ε 4·93), 257 (4·94), and 301 nm (4·62), $\nu_{max.}$ 2950, 1730, 1585, 1140, 1050, and 840 cm^{-1} (Si–C), δ 0·18 (9H, s, $SiMe_3$), 1·48 (12H, s, 2′-Me_2, 3″-Me_2), 3·80 (3H, s, OMe), 6·65 and 5·69 (2H, ABq, *J* 10 Hz, H-4′, H-3′), 6·95 and 6·81 (2H, ABq, *J* 16 Hz, H-1″, H-2″), and 6·27, 8·06 (2H, ABq, *J* 10 Hz, H-3 and -4), *m/e* 414 (M^+, 24%), 399 ($C_{22}H_{27}O_5Si$, 100), 371 (12), 279 (21), and 180 (60).

Catalytic Hydrogenation of Avicennol.—Avicennol (200 mg) in absolute ethanol (100 ml) was hydrogenated for 10 h at room temperature and atmospheric pressure over Adams catalyst (20 mg). The mixture was then filtered; t.l.c. on alumina (as already described) indicated two compounds, R_F 0·4 (A) and 0·7 (B), both showing a blue fluorescence in screened u.v. light (366 nm). Separation was achieved by preparative t.l.c. on neutral alumina (Woelm activity I) with chloroform–ethanol (98 : 2) as eluant. The products were extracted from the bands with chloroform.

Compound (A) was recrystallised from methanol to give *tetrahydroavicennol* (9) as prisms, m.p. 165—165·5° (Found: M^+, 346·1779. $C_{20}H_{26}O_5$ requires *M*, 346·1780), $\lambda_{max.}$ 213 (log ε 4·42), 258 (3·84), and 316 nm (4·09), $\nu_{max.}$ 3420 (OH), 2940, 1710 (CO), 1600, and 1115 cm^{-1}, δ 1·35 (6H, s, Me_2), 1·39 (6H, s, Me_2), 1·83 (4H, m, 2″- and 3′-H_2), 1·96 (1H, s, exchanged with D_2O, OH), 2·83 (4H, m, 1″- and 4′-H_2), 3·90 (3H, s, OMe), and 6·27 and 8·12 (2H, ABq, *J* 10 Hz, H-3 and -4), *m/e* 346 (M^+, 21%), 331 (6), 329 (15), 328 (66), 313 (12), 273 ($C_{16}H_{17}O_4$, 100), and 217 (51).

Compound (B) was recrystallised from light petroleum (b.p. 40—60) to give hexahydroavicennin (10) as white

* Voucher Specimen No. STRATHCLYDE 30 has been deposited at the Herbarium of the Royal Botanic Garden, Edinburgh.

prisms, m.p. 81—82° (lit.,[1c] 81—82°) (Found: M^+, 330·1824. Calc. for $C_{20}H_{26}O_4$: M, 330·1831), $\lambda_{max.}$ 217, 257, and 315 nm, $\nu_{max.}$ 2920, 1725 (CO), 1600, and 1120 cm^{-1}, *m/e* 330 (M^+, 91%), 275 (29), 274 (35), 273 ($C_{16}H_{17}O_4$, 100), and 217 (23), n.m.r. spectrum identical with that previously recorded.[2]

Avicennin (2).—Avicennol (100 mg) was dissolved in benzene (sodium-dried) and a 12·5% solution (0·25 ml) of phosgene in toluene added. After 3 h the excess of phosgene was degraded by addition of absolute ethanol (1 ml). The mixture was evaporated to dryness to yield a solid which was recrystallised from methanol to give avicennin as yellow needles, m.p. 138—140° (lit.,[3] m.p. 136·5—138·5°) (Found: M^+, 324·1359. $C_{20}H_{20}O_4$ requires M, 324·1361), $\lambda_{max.}$ 270, 278, and 301 nm, $\nu_{max.}$ 2940, 1730 (CO), 1585, and 1140 cm^{-1}, n.m.r. spectrum identical with that previously recorded.[2]

N.m.r. Shift Experiments.—All experiments were carried out with $CDCl_3$ as solvent, tetramethylsilane as internal standard, and $Eu(fod)_3$ as shift reagent. The weighed sample (60—80 mg) was dissolved (0·3—0·5 ml solvent) and the spectrum recorded. The shift reagent was then added in weighed quantities of *ca.* 10 mg, at least four spectra being recorded for each compound, each with a larger quantity of shift reagent. Graphs were then plotted of shift (Hz) *versus* weight of $Eu(fod)_3$ added to find shifts for all protons, which were then expressed [8] relative to that of H-3 (= 1·00). The results were as follows: avicennol (4) H-3 (1·00), H-4 (0·40), H-2″ (2·56), H-1″ (2·08), MeO (0·40), H-4′ (0·26), H-3′ (0·14), 2′-Me_2 (0·14), 3″-Me_2 (1·59), OH (8·57); 3″-O-trimethylsilylavicennol (5) H-3 (1·00), H-4 (0·31), H-2″ (0·52), H-1″ (0·40), MeO (0·15), H-4′ (0·13), H-3′ (0·08), 2′-Me_2 (0·08), 3″-Me_2 (0·13); avicennin (2) H-3 (1·00), H-4 (0·29), H-2″ (0·38), H-1″ (0·41), MeO (0·13), H-4′ (0·12), H-3′ (0·08), 2′-Me_2 (0·08), 3″-Me (0·09), 4″-H_2 (0·03, 0·09); hexahydroavicennin (10) H-3 (1·00), H-4 (0·30), 2″-H_2 (0·24), 1″-H_2 (0·36), MeO (0·13), 4′-H_2 (0·13), 3′-H_2 (0·11), 2′-Me_2 (0·09), 3″-Me_2 (0·11).

We thank Dr. R. A. Spragg, Perkin-Elmer, Beaconsfield, Bucks., for carrying out the n.O.e. measurements, Mr. S. P. Lau, Agriculture and Fisheries Department, Govt. of Hong Kong, for the collection and identification of plant material, and the University of Strathclyde for a grant (to A. I. G.).

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DIPETALOLACTONE: A NOVEL PYRANOCOUMARIN FROM THE ROOT BARK OF *ZANTHOXYLUM DIPETALUM*

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Key Word Index—*Zanthoxylum dipetalum*; Rutaceae; pyranocoumarins; dipetalolactone; tetrahydrodipetalolactone; dipetaline; synthesis.

Abstract—A novel dipyranocoumarin, dipetalolactone {2-oxo-6,6,10,10-tetramethylbenzo[1,2-b:3,4-b′:5,6-b″]tripyran}, has been isolated from the root bark of *Zanthoxylum dipetalum* and its structure proven by the synthesis of tetrahydrodipetalolactone. A second new pyranocoumarin, dipetaline, has been assigned the tentative structure of 6-(3,3-dimethylallyl)-5-methoxy-2,2-dimethyl-2H-benzo[1,2-b:3,4-b′]dipyran-8-one on the basis of PMR analysis using the lanthanide shift reagent $Eu(fod)_3$.

INTRODUCTION

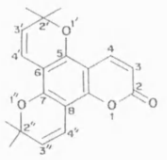
In a previous publication [1] we reported the isolation of the alkaloids canthin-6-one, chelerythrine, nitidine, tembetarine and magnoflorine, the pyranocoumarins avicennol and xanthoxyletin, the triterpenes lupeol and sitosterol, and the flavanone hesperidin from the Hawaiian tree *Zanthoxylum dipetalum* H. Mann (Rutaceae). A third pyranocoumarin (designated ZD/1), isolated only in small amounts and in an impure state, was tentatively assigned a unique dipyranocoumarin nucleus, largely on the basis of MS data.

We now report the complete structure elucidation of this novel coumarin, to which we give the trivial name dipetalolactone (**1**), and the synthesis of its tetrahydro derivative. In addition it has been shown that the contaminant preventing the crystallisation of dipetalolactone from ZD/1 is another pyranocoumarin. This compound, which we have called dipetaline, is tentatively identified as the angular pyrano[2,3-f]coumarin (**8**) which is closely related to avicennol, the major coumarin of this species.

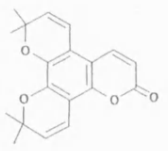
RESULTS AND DISCUSSION

The petrol extract of the root bark of *Z. dipetalum*, after deposition of avicennol, was subjected to repeated PLC over alumina to give 65 mg of a yellow oil designated ZD/1 [1]. Although exhibiting only a single spot on TLC this material was shown (by PMR) to be a mixture of two coumarins in the approximate ratio of 4:1. Repeated recrystallisation of the oil from absolute EtOH gave dipetalolactone (46 mg) mp 119–120°. The MS, UV and IR, reported previously [1], were all typical of pyranocoumarins [2]. Accurate mass measurement gave a molecular ion M^+ 310·1206 ($C_{19}H_{18}O_4$) and a base peak at *m/e* 295 for loss of $Me^\cdot$. Further loss of $Me^\cdot$ from the ion at *m/e* 295 gave a doubly charged fragment at *m/e* 140.

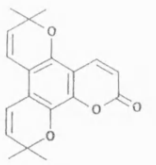
The PMR spectrum readily resolved all eighteen protons of dipetalolactone. An AB quartet (J 10 Hz) δ 6·19 (1H) and 8·02 (1H) confirmed the presence of the α,β-unsaturated lactone ring of the coumarin nucleus [2]. The presence of two of the 2,2-dimethylchromene ring systems encountered in pyranocoumarins [3,4] was indicated by the occurrence of a singlet for *gem*-methyl groups at δ 1·48 (12H) and two AB quartets (both J 10 Hz) centered at δ 6·88 (1H), 5·65 (1H) and δ 6·71 (1H), 5·62 (1H). This easily rationalised PMR spectrum confirmed that the major component of the oil ZD/1 had the previously suggested [1] dipyranocoumarin structure but failed to distinguish between the four possible isomers (**1**–**4**).



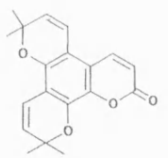
Dipetalolactone (1)



(2)



(3)



(4)

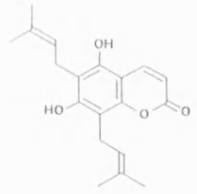
Of the possible isomeric structures (**1**) was considered the most likely in view of the following. Firstly, almost all naturally occurring coumarins are oxygenated in the

* To whom communications should be addressed.

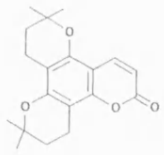
7-position* [5], thereby requiring that the C-4″ attachment be in the 8- position, as in structures (**1**) and (**2**). Secondly, avicennol and xanthoxyletin, the two coumarins already reported from this species, both exhibit a 5,7-oxygenation pattern [cf. (**1**)].

A significant contribution to the assignment of structure (**1**) to dipetalolactone was made by PMR studies using the lanthanide shift reagent Eu(fod)$_3$ (tris-(7,7-dimethyl-1,1,1,2,2,3,3-heptafluoro-octane-4,6-dionato)europium) according to a method described elsewhere [6]. Complexation proceeded at the carbonyl group as expected with the observed shift of the 4-H (relative to 3H = 1·00) as before [7]. However other shifts, particularly that of the putative C-8 pyran substituent, were less than the calculated values. This may be explained by a preferred out-of-plane conformation for the lactone ring due to interaction of the 4″-H with the electron clouds of the lactone oxygen and thereby resulting in the formation of a non-coplanar complex [8]. Despite the variation of shift values obtained from those observed previously [6,7] the results obtained still gave a 'best-fit' for, and could only be assigned to, structure (**1**).

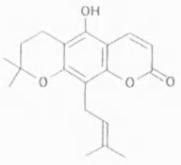
In order to confirm the structure of dipetalolactone its tetrahydro derivative (**6**) was synthesised. The nuclear prenylation of 5,7-dihydroxy-coumarin, in which one of the major products was 5,7-dihydroxy-6,8-di-(3,3-dimethylallyl)-coumarin (**5**), has been described [9]. Acid-catalysed cyclisation of an *ortho*-(3,3-dimethylallyl)-hydroxycoumarin (osthenol) to the corresponding dihydropyranocoumarin (dihydroseselin) has also been reported [10]. These synthetic steps provide a convenient method for the preparation of 2-oxo-6,6,10,10-tetramethyl-7,8,11,12-tetrahydro-benzo[1,2-b:3,4-b′:5,6-b″]tripyran (**6**), although the alternative cyclisation to the linear dihydropyranocoumarin dihydrotrachyphyllin (**7**) might also occur. Refluxing (**5**) with formic acid and subsequent PLC gave (**6**) mp 154–155° as the major product with minor amounts of a second compound, possibly (**7**).



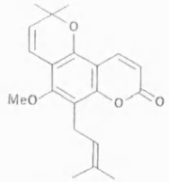
(**5**)



(**6**)



(**7**)



Dipetaline (**8**)

* In order to clarify the text, the non-systematic numbering system illustrated in formula (**1**) is used to refer to positions on the dipetalolactone skeleton.

Hydrogenation of dipetalolactone over Adam's catalyst yielded colourless plates, mp 154–155°, undepressed on admixture with (**6**). Tetrahydrodipetalolactone and (**6**) were found to be identical in all respects (UV, IR, PMR, MS) thereby confirming the structure of dipetalolactone as (**1**). A lanthanide shift experiment with tetrahydrodipetalolactone gave essentially the same results as dipetalolactone.

The minor coumarin, dipetaline, which remained in a 1:1 mixture with dipetalolactone in the supernatant after the deposition of the latter, was not further purified. MS of the mixture indicated a molecular formula for dipetaline of $C_{20}H_{22}O_4$ with a major ion at *m/e* 311 for the loss of Me$^{\cdot}$. PMR of the mixture permitted all twenty-two protons of dipetaline to be distinguished from those of dipetalolactone. An AB quartet (*J* 10 Hz) at δ 6·30 (1H) and 8·10 (1H) demonstrated the presence of an α,β-unsaturated lactone ring. A second AB quartet (*J* 10 Hz) at δ 5·71 (1H) and 6·64 (1H) together with a singlet for *gem*-methyl groups at δ 1·48 (6H) was again indicative of the 2,2-dimethylchromene ring system of pyranocoumarins. The presence of a methoxy substituent was shown by a singlet at δ 3·85 (3H) whilst broad singlets at δ 1·72 (3H) and 1·87 (3H) together with multiplets at δ 3·50 (2H) and 5·33 (1H) could be accounted for by a 3,3-dimethylallyl unit. Therefore dipetaline is a pyranocoumarin with the two remaining positions on the benzenoid nucleus filled by methoxy and 3,3-dimethylallyl substituents, respectively.

In an attempt to determine the substitution pattern of dipetaline it was examined using Eu(fod)$_3$. Addition of Eu(fod)$_3$ to the mixture allowed, in the light of previously obtained shift values for the protons of dipetalolactone, the determination of the shift for the protons of dipetaline (relative to 3H = 1·00). In this case 'best-fit' was obtained between observed and calculated shifts for values of ϕ (C–O–Eu bond angle) of 152·5° and *d* (O–Eu distance) of 2·75Å, in agreement with our previous findings [6,7]. The large observed shifts for the allylic protons (Table 1) indicated the placement of the 3,3-dimethylallyl unit in the 8-position. The observed methoxy shift (0·15) suggested its placement in the 7-position (calculated shift 0·15) rather than the 5-position (calculated shift 0·11). Furthermore observed and calculated shifts for the pyran ring methyl protons offer marginally better fits with pyran ring attachment through the oxygen at C-5 rather than that at C-7 and therefore also support the angular structure (**8**) for dipetaline. Circumstantial evidence in favour of this assignment for dipetaline is

Table 1. Observed and calculated shift values for dipetaline with Eu(fod)$_3$ (relative to 3H = 1·00)

(a) for the allylic protons

	observed	calculated for attachment at			
		C-5	C-6	C-7	C-8
CH_2*	0·35	0·15	0·12	0·15	0·32
CH*	0·54	0·17	0·12	0·16	0·48

(b) for the methoxy and pyran ring methyl groups

	observed	calculated for attachment to oxygen at	
		C-5	C-7
OMe	0·15	0·11	0·15
Me_2	0·08	0·08	0·09

* The calculated values are the average of the two extreme conformations of the side-chain in the plane of the coumarin benzene ring with the allylic protons *trans*-orientated.

provided in its obvious relationship to avicennol, the major coumarin of *Z. dipetalum*.

The presence of several derivatives of 5,7-dihydroxy-6,8-(3,3-dimethylallyl)coumarin in *Z. dipetalum* is of systematic interest. A similar range of 5,7-dihydroxy-6,8(3,3-dimethylallyl)-2-methylchromones, including the dipyranoid compound spatheliabischromene, occur in the small aberrant genus *Spathelia* L. [11]. The co-occurrence of this unusual type of substitution might be considered to offer further chemical support to that already available [12] in favour of the retention of *Spathelia* in the Rutaceae and against its transfer to the Simaroubaceae. Other dipyranoid substituents have been reported among the acridone alkaloids of the rutaceous genus *Atalantia* Correa [13] and among the chromones of *Cneorum tricoccum* L. [14] of the closely allied family Cneoraceae.

EXPERIMENTAL

UV spectra were recorded in EtOH and IR spectra in KCl. PMR (60 MHz) were recorded in $CDCl_3$ with TMS as internal standard. MS were recorded on an AEI MS902 spectrometer at 70 eV. Mp's (uncorr) were determined on a Kofler hot stage. PLC were carried out on 1 mm thick layers eluting with $CHCl_3$.

Plant material. Root bark of *Zanthoxylum dipetalum* H. Mann (Voucher: GS-8 at BISH, HLA and NBV [15]) was collected at the Pupukea-Paumalu Forest Reserve, Koolau Mountains, Oahu Hawaii.

Extraction and isolation. Milled bark (55 g) was extracted in a Soxhlet with petrol (bp 40–60°) and the extract conc under red. pres. On standing a ppt. of avicennol (560 mg) was obtained [1]. An aliquot of the supernatant (50%) was subjected to PLC on alumina (Woelm, neutral, activity I) with *n*-hexane–EtOAc as eluting solvent. Xanthoxyletin and lupeol were isolated and identified as previously described [1]. A third yellow band, designated ZD/1 (R_f 0·5, 10 mg), was eluted and on careful re-PLC of adjacent alumina bands a further 15 mg of this material was obtained. The remainder of the petrol extract was similarly treated by PLC on Si gel G, eluting with $CHCl_3$, to give further ZD/1 (R_f 0·5, 45 mg) together with canthin-6-one. Repeated PLC of the combined ZD/1 fractions over alumina gave 65 mg, as a yellow oil, which was shown, by PMR ($CDCl_3$), to be a mixture of two coumarins.

Dipetalolactone (**1**). Recrystallisation of the mixture ZD/1 from absolute EtOH gave, after several days, yellow plates, mp 119–120°. Found M$^+$ 310·1206, $C_{19}H_{18}O_4$ requires 310·1205. UV λ_{max} nm (log ϵ): 222(4·17), 244*sh*(4·37), 250(4·47), 294*sh*(4·37), 297(4·38), 307*sh*(4·28), 344(4·04); absence of bathochromic shift on addition of NaOH solution. IR ν_{max} cm^{-1}: 2990, 1730(C=O), 1610, 1360, 1135, 1025, 825, 740, 705. MS *m/e*: 310(35%), 295(100), 140(M^{2+} ion). PMR δ: 1·48 (12H, *s*, 2′-Me_2, 2″-Me_2), 5·62, 6·71 (2H, AB*q*, *J* 10 Hz, 3′-H, 4′-H†), 5·65, 6·88 (2H, AB*q*, *J* 10 Hz, 3″-H, 4″-H†), 6·19, 8·02 (2H, AB*q*, *J* 10 Hz, 3-H, 4-H).

Tetrahydrodipetalolactone (**6**). Hydrogenation of dipetalolactone (30 mg) over Adam's catalyst (20 mg) in EtOH (absolute), hydrogen uptake occurring over 4 hr, gave, on filtration and recrystallisation from MeOH, tetrahydrodipetalolactone (25 mg) as colourless plates mp 154–155°. Found M$^+$ 314·1514, $C_{19}H_{22}O_4$ requires 314·1518. UV λ_{max} nm (log ϵ): 211(4·55), 225*sh*(4·13), 253(3·80), 262(3·85), 337·5(4·16). IR ν_{max} cm^{-1}: 3000, 2950, 1730(C=O), 1620, 1380, 1165, 825, 820. MS *m/e*: 314 (80%), 259(100), 258(56), 243(23), 203(76). PMR δ: 1·37 (12H, *s*, 2′-Me_2, 2″-Me_2), 1·80, 2·64 (4H, 2 x *tr*, *J* 7 Hz, 3′-CH_2, 4′-CH_2†), 1·83, 2·85 (4H, 2 x *tr*, *J* 7 Hz, 3″-CH_2, 4″-CH_2), 6·16, 8·10 (2H, AB*q*, *J* 10 Hz, 3-H, 4-H).

† Assignments of pyran ring signals in the PMR spectra of (**1**) and (**6**) were made on the basis of observed shifts with Eu(fod)$_3$.

Synthesis of 2-oxo-6,6,10,10-tetramethyl-7,8,11,12-tetrahydrobenzo[1,2-b:3,4-b′:5,6-b″]tripyran (**6**). *Preparation of 5,7-dihydroxycoumarin.* Perkin condensation [16] of phloroglucinaldehyde with Ac_2O in the presence of anhydrous NaOAc gave 5,7-diacetoxycoumarin, recrystallised from EtOH as prisms mp 140° (lit. [16] 140°). The previously reported [16] alkaline hydrolysis of 5,7-diacetoxycoumarin resulted in an intense red solution from which no 5,7-dihydroxycoumarin could be recovered. Acid hydrolysis of 5,7-diacetoxycoumarin was achieved by heating a conc ethanolic solution in 50% HCl for 4 hr at 100°C. Evaporation of the EtOH gave 5,7-dihydroxycoumarin, recrystallised from dil AcOH as needles (83% yield) mp 283–285° (lit. [16] 285–286°).

Preparation of 5,7-dihydroxy-6,8-di-(3,3-dimethylallyl)coumarin (**5**). Reaction of 5,7-dihydroxycoumarin with 2-methylbut-3-en-2-ol by the method of Mahey *et al.* [9] afforded an oil having identical characteristics (UV, IR, PMR) to those recorded for 5,7-dihydroxy-6,8-di-(3,3-dimethylallyl)coumarin [9].

Preparation of 2-oxo-6,6,10,10-tetramethyl-7,8,11,12-tetrahydrobenzo[1,2-b:3,4-b′:5,6-b″]tripyran (**6**). 5,7-Dihydroxy-6,8-di-(3,3-dimethylallyl)coumarin (60 mg) was heated on a water bath with formic acid for 4 hr. The reaction mixture was diluted with H_2O and extracted with CH_2Cl_2. Concn of the CH_2Cl_2 extract followed by PLC over alumina, eluting with $CHCl_3$ (blue fluorescent band, R_f 0·63), gave, on recrystallisation from MeOH, colourless plates (25 mg) mp 154–155° identical in all respects (UV, IR, PMR, MS, TLC, mmp) with tetrahydrodipetalolactone.

Dipetaline (**8**). The supernatant EtOH solution after deposition of dipetalolactone was subjected to MS and PMR analysis. The following data were obtained after comparison with spectra of pure dipetalolactone. Found M$^+$ 326·1513, $C_{20}H_{22}O_4$ requires 326·1518. MS *m/e*: 326, 311. PMR δ: 1·48 (6H, *s*, pyran-Me_2), 1·72 (3H, *s*, allylic Me), 1·87 (3H, *s*, allylic Me), 3·50 (2H, *br. d.*, allylic CH_2), 3·85 (3H, *s*, OMe), 5·33 (1H, *br. tr.*, allylic CH), 5·71, 6·64 (2H, AB*q*, *J* 10 Hz, pyran CH:CH), 6·30, 8·10 (2H, AB*q*, *J* 10 Hz, 3-H, 4-H).

Acknowledgements—We thank Mr. J. T. Hirano and Mr. G. Spence of the Lyons Herbarium, University of Hawaii, for the supply and authentication of plant material. One of us (A.I.G.) wishes to thank the University of Strathclyde for the award of a scholarship.

REFERENCES

1. Fish, F., Gray, A. I. and Waterman, P. G. (1975) *Phytochemistry* **14**, 2073.
2. Neilsen, B. E. (1970) *Dan. Tidsskr. Farm.* **44**, 161.
3. Arthur, H. R. and Ollis, W. D. (1963) *J. Chem. Soc.* 3910.
4. Tomimatsu, T., Hashimoto, M., Shingu, T. and Tori, K. (1969) *J. Chem. Soc., Chem. Commun.* 168.
5. Dean, F. M. (1963) *Naturally Occurring Oxygen Ring Compounds*, p. 176, Butterworths, London.
6. Gray, A. I., Waigh, R. D. and Waterman, P. G. (1974) *J. Chem. Soc. Chem. Comm.*, 632.
7. Gray, A. I., Waigh, R. D. and Waterman, P. G. (1975) *J. Chem. Soc. Perkin Trans.* I, 488.
8. Gray, A. I., Waigh, R. D. and Waterman, P. G. Work on the non-conforming shift values obtained for pyrano[3,2-h]coumarins is in progress and will be published later.
9. Mahey, S., Seshadri, T. R. and Mukerjee, S. K. (1974) *Indian J. Chem.* **12**, 29.
10. Bottomley, W. and White, D. E. (1951) *Austral. J. Sci. Res. A.* **4**, 12.
11. Taylor, D. R. and Wright, J. A. (1971) *Rev. Latinoamer. Quim.* **2**, 84.
12. Adams, C. D., Taylor, D. R. and Warner, J. M. (1973) *Phytochemistry* **12**, 1359.

13. Basu, D. and Basa, S. C. (1972) *J. Org. Chem.* **37**, 3035.
14. Gonzalez, A. G., Fraga, B. M. and Ano, O. (1974) *Phytochemistry* **12**, 956.
15. Lanjouw, J. and Staflau, F. A. (1964) *Index Herbariorum*, 5th Ed., Part I, I.A.P.T., Utrecht, Netherlands.
16. Heyes, R. G. and Robertson, A. (1936) *J. Chem. Soc.* 1831.

dewevrei (*C. dewevrei* De Wild. et Durand var. *excelsa* Chev. and *C. dewevrei* De Wild. et Durand var. *arnowimiensis* (De Wild.) Chev.).

The dried and finely ground leaf material (portions of 1 g) was boiled in 125 ml 0.01 n H_2SO_4 (20 min), mixed with 13 g MgO, cooled and filtered through glass filter G4. The filtrate was extracted with $CHCl_3$ (100 ml × 3). Following evaporation of $CHCl_3$, the concentrate was chromatographed by preparative TLC on Si gel ($CHCl_3$–MeOH 9:1). The zone with R_f 0.52 was eluted with MeOH and rechromatographed. Crystallization from MeOH (and few drops of H_2O) yielded needles (mp 202°) with mass, UV and IR spectra [1] identicl to those of the authentic sample of **3** with mp 205° prepared by methylation of 7,9-dimethyluric acid [3,4]. Thermal rearrangement of both the natural and synthesized sample gave 1,3,7,9-tetramethyluric acid (**1**).

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REFERENCES

1. Wanner, H., Pešáková, M., Baumann, T. W., Charubala, R., Guggisberg, A., Hesse, M. and Schmid, H. (1975) *Phytochemistry* **14**, 747.
2. Baumann, T. W., Occhslin, M. and Wanner, H. (1976) *Biochem. Physiol. Pflanz.* **170**, 217.
3. Biltz, H. and Bülow, H (1921) *Ann. Chem.* **423**, 159.
4. Biltz, H. und Max, F. (1920) *Ber. Deut. Chem. Ges.* **53**, 2327.

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CHONDROFOLINE FROM *UVARIA OVATA**

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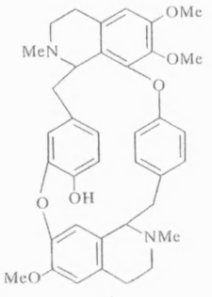
Key Word Index—*Uvaria ovata*: Annonaceae; chondrofoline; *bis*-1-benzyltetrahydroisoquinoline alkaloid.

Chondrofoline, a member of the rare group of 7,3‴–8′,4″-linked *bis*-benzylisoquinolines, has been found in *Uvaria ovata* (Annonaceae); it has previously been found only in *Chondrodendron platyphyllum* Miers (Menispermaceae) [1]. Alkaloids of this type have previously been isolated only from Menispermaceae and Lauraceae [2] and their discovery in another, closely allied, Ranalean family yet again illustrates the potential value of alkaloids in the systematics of the Ranales.

EXPERIMENTAL

Plant. Uvaria ovata A. DC; Voucher. Enti 1284, deposited at the herbarium of the Royal Botanic Garden, Edinburgh; Source. Achimota, Ghana.

Alkaloid isolation. Powdered leaf (650g) was extracted successively with petrol (40–60°), $CHCl_3$ and MeOH. Acid extraction of the $CHCl_3$ concn., basification of the acid extract with NH_3 and re-extraction into $CHCl_3$ gave a mixture of alkaloids. Col. chr. of the mixture over Al gave, on elution with $CHCl_3$–MeOH (99:1), a single alkaloid. Recrystallisation of the alkaloid from $CHCl_3$–Et_2O and finally Et_2O gave plates (97 mg) mp 136–140°. $[\alpha]_{546}^{20}$ − 257 (*c* 0.10 in 0.1 N HCl). Found, M^+ 608.2877; $C_{37}H_{40}N_2O_6$ requires 608.2886. UV λ_{max}^{EtOH} nm (log ε) 232 (4.57), 281 (3.97), undergoing a bathochromic shift on the addition of alkali. IR ν_{max} (KBr) cm^{-1} 3450 (OH). PMR ($CDCl_3$) δ 2.30 (3H, *s* N-Me), 2.58 (3H, *s* N-Me), 3.78 (3H, *s* OMe), 3.92 (6H, *s* 2 × OMe), 2.60–3.85 (14H, *m* CH_2 and CH), 6.00 (1H, *s* C-8′-H), 6.65–7.35 (9H, *m* Har). MS 608 (91), 607 (50), 487 (2), 312 (92), 311 (19), 299 (24), 298 (100), 266 (10), 204 (23), 192 (12), 190 (15), 176 (11.5), 174 (19), 161 (6.5), 159 (10), 146 (11), 145 (15). From UV, IR and PMR spectra and accurate mass measurement of the molecular ion, it appeared likely that the alkaloid was of the *bis*-1-benzyltetrahydroisoquinoline type, with one OH, 3 × OMe and 2 × NMe substituents. The significance of MS fragmentation patterns in the identification of *bis*-benzylisoquinoline alkaloids and the probable origin of the fragments observed have been thoroughly discussed [3]. The major ions, at *m/e* 312 ($C_{19}H_{22}NO_3$) and *m/e* 298 ($C_{18}H_{20}NO_3$), indicated that the two benzylisoquinoline moieties were linked head to tail. In addition, the relatively high abundance of an ion at *m/e* 204 ($C_{12}H_{14}NO_2$) suggested the presence of a 6,7-dimethoxyisoquinoline fragment, and the relatively low abundance of an ion corresponding to loss of Me from the other benzylisoquinoline subunit indicated MeO substitution in this isoquinoline unit rather than in the benzyl group attached to it. A detailed comparison of the complete MS with those of known head to tail linked *bis*-benzylisoquinolines [4] suggested that the alkaloid



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* Part 3 in the series 'Chemical Studies on the Annonaceae'. For Part 2 see Panichpol, K., Waigh, R. D. and Waterman, P. G. (1976) *J. Pharm. Pharmacol.* **28**, 71p.

was chondrofoline (1) or a stereoisomer thereof. The stereochemical coidentity of the alkaloid from *U. ovata* was ascertained by comparison of the ORD spectrum [1, 5] with that of authentic chondrofoline and the structure was finally confirmed by direct comparison (UV, IR, mmp, TLC).

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REFERENCES

1. Bick, I. R. C., Baldas, J., Porter, Q. N. and Vernengo, M. J. (1971) *Chem. Commun.* 132.
2. Kametani, T. (1969) *The Chemistry of the Isoquinoline Alkaloids*. Elsevier, London.
3. Baldas, J., Bick, I. R. C., Ibuka, T., Falco, M. R., de Vries, J. X. and Porter, Q. N. (1972) *J. Chem. Soc. Perkin Trans. I*, 592.
4. Baldas, J., Bick, I. R. C., Ibuka, T., Kapil, R. S. and Porter, Q. N. (1972) *J. Chem. Soc.* suppl. publ. No. SUP 20264.
5. Battersby, A. R., Bick, I. R. C., Klyne, W., Jennings, J. P., Scopes, P. M. and Vernengo, M. J. (1965) *J. Chem. Soc.* 2239.

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PHENETHYLAMINES FROM *ECHINOCEREUS CINERASCENS* AND *PILOSOCEREUS CHRYSACANTHUS**

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Key Word Index—*Echinocereus cinerascens*; *Pilosocereus chrysacanthus*; Cactaceae; alkaloids; *N,N*-dimethyl-3,4-dimethoxyphenethylamine; *N*-methyl-3,4-dimethoxyphenethylamine; *N*-methyl-3,4-dimethoxyphenethylamine

In a field screening of Mexican cacti for the presence of alkaloids, *Echinocereus cinerascens* (DC.) Rümpler and *Pilosocereus chrysacanthus* (Web.) Byl. et Rowl. were found to give positive tests with the Dragendorff reagent [1]. Plants were collected and the alkaloids extracted and studied. The present report describes the isolation and identification of the major phenethylamine alkaloids of these two cactus species.

Although several alkaloid screening papers have listed various *Echinocereus* species [2–4], only one species, *E. merkeri*, has been investigated in more detail. *N,N*-dimethyl-3,4-dimethoxyphenethylamine was isolated for the first time from *E. merkeri*, which contains several additional phenethylamines and the tetrahydroisoquinoline salsoline [5, 6].

We have now identified the major alkaloid of *E. cinerascens* as *N,N*-dimethyl-3,4-dimethoxyphenethylamine. Alkaloid extraction followed by fractionation on an alumina column led to the isolation of this compound, as well as small amounts of *N*-methyl-3,4-dimethoxyphenethylamine. *E. cinerascens* has an edible fruit [7] and dry plants are used as fuel [8], but no medicinal uses seem to have been recorded for *E. cinerascens* or *Pilosocereus chrysacanthus*. The major alkaloid of the latter species was identified as *N*-methyl-3,4-dimethoxyphenethylamine.

The alkaloids now isolated were identified by comparison with synthetic reference materials using TLC, GC, IR, and MS. A part of the *N,N*-dimethyl-3,4-dimethoxyphenethylamine isolated from *E. cinerascens* was oxidized to the corresponding 3,4-dimethoxybenzoic acid, identified by IR and mp comparison with an authentic sample.

N-Methyl- and *N,N*-dimethyl-3,4-dimethoxyphenethylamine have been reported from *E. merkeri* [5], and are also found in other genera of the Cactaceae, e.g. *Coryphantha* [9] and *Ariocarpus* [10].

EXPERIMENTAL

Plant material. Echinocereus cinerascens (DC.) Rümpler (4.1 kg) was collected north of Pachuca, Hidalgo, and *Pilosocereus chrysacanthus* (Web.) Byl. et Rowl. (3.0 kg) near San Antonio Texcala, Puebla, by the authors.

Alkaloid extraction. Fresh plant material was homogenized in EtOH. The filtered extracts were evaporated to dryness and dissolved in 3% HOAc. The aq. phases were extracted with $CHCl_3$ and the $CHCl_3$ discarded. Aq. phases were basified with NH_3 conc (pH 10) and alkaloids extracted with $CHCl_3$ and $CHCl_3$–EtOH (3:1). Crude alkaloids were purified on an acid diatomaceous earth column (Celite 545). Yield of alkaloids: *E. cinerascens* 585 mg; 0.014%; *P. chrysacanthus* 684 mg; 0.02%.

Isolation and identification. The alkaloid extract of *E. cinerascens* (525 mg) was fractionated on an aluminium oxide column (Merck, act. II–III acc. to Brockmann) as earlier described [5]. The eluates were analyzed by TLC and GC (5% SE-30 and 5% XE-60 columns, col. temp. 150°) [11]. MS were obtained with a combined GC–MS instrument (ion source 2.5 kV, electron energy 70 eV, and ionization current 60 μA). *N,N*-Dimethyl-3,4-dimethoxyphenethylamine was eluted with $CHCl_3$–C_6H_6 (1:2) and crystallized as the hydrochloride (292 mg) mp 193–197°; lit. mp 194–196° [4]. Alkaline permanganate oxidation of 50 mg of this compound gave 10 mg of 3,4-dimethoxybenzoic acid, mp 178–181°; lit. mp 181° [12]. *N*-methyl-3,4-dimethoxyphenethylamine was isolated from the $CHCl_3$–MeOH (4:1) fractions as the hydrochloride (yield 8 mg), mp 134–136°; lit. mp 136–137° [4]. Preparative TLC on Si gel GF plates in $CHCl_3$–EtOH–NH_3 conc (80:20:0.4) of 80 mg of the *P. chrysacanthus* alkaloids yielded *N*-methyl-3,4-dimethoxyphenethylamine, which was crystallized as the hydrochloride (yield 21 mg), mp 134–135°; lit. mp 136–137° [4].

*Cactaceae Alkaloids. 27.

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CIS-AVICENNOL, A NEW PYRANOCOUMARIN FROM THE ROOT BARK OF *ZANTHOXYLUM ELEPHANTIASIS*

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Key Word Index—*Zanthoxylum elephantiasis*; Rutaceae; pyranocoumarin; *cis*-avicennol; tetrahydroavicennol; lanthanide shift reagent $Eu(fod)_3$.

Abstract—A new pyranocoumarin has been isolated from the root bark of *Zanthoxylum elephantiasis* and identified as *cis*-avicennol (6-(3-hydroxy-3-methyl-*cis*-but-1-enyl)-5-methoxy-2,2-dimethyl-2*H*-benzo [1,2b:3,4b'] dipyran-8-one) on the basis of comparison of spectral data with that of *trans*-avicennol and conversion to tetrahydroavicennol. The usefulness of the lanthanide shift reagent $Eu(fod)_3$ in the assignment of *cis* configuration to the butenyl side-chain is briefly discussed.

INTRODUCTION

The angular pyranocoumarin avicennol (1) has, to date, been found to occur only in three species of the rutaceous genus *Zanthoxylum* L.: *Z. avicennae* (Lam.) DC. [1], *Z. dipetalum* Mann [2] and *Z. elephantiasis* Macfad. [3]. In the determination of the structure of avicennol [1] considerable use was made of the shifts induced in the PMR spectrum of its *O*-TMSi ether by the lanthanide shift reagent $Eu(fod)_3$. Subsequently the structure of avicennol, with its unusual 3-hydroxy-3-methyl-*trans*-but-1-enyl side-chain, was confirmed by synthesis [4].

We now wish to report the isolation of the corresponding *cis* isomer of avicennol from the root bark of *Z. elephantiasis* and its identification by comparison of spectral data and shift values with $Eu(fod)_3$ with those previously obtained for *trans*-avicennol.

RESULTS AND DISCUSSION

On concentration the petrol extract of the root bark of *Z. elephantiasis* deposited, in high yield, avicennol, xanthoxyletin and canthin-6-one. PLC of the supernatant liquor gave, in addition, dihydrochelerythrine, avicennin, dipetalolactone and triacontanoic acid [3]. Concentration of the supernatant solution from which triacontanoic acid had been obtained yielded 95 mg of a yellow oil, fluorescing dull yellow under UV. The yellow oil, although giving a single spot on TLC using several systems, could not be obtained in crystalline form.

MS of the oil indicated a molecular ion M^+ 342.1453 ($C_{20}H_{22}O_5$) with a fragmentation pattern showing only relative abundance variations from that observed for *trans*-avicennol [1]. Likewise, the IR spectrum, apart from a distinct broadening of the OH band, was in close agreement with that previously recorded for *trans*-avicennol.

Conversely the maxima observed in the UV spectrum of the oil (236, 275 and 294 nm; cf. 250, 257 and 301 nm for *trans*-avicennol) and the chemical shifts of some of the protons in the PMR (Table 1) showed distinct differences. With respect to the PMR spectrum, whilst the resonance signals were indicative of a coumarin with similar substituents to *trans*-avicennol, differences in chemical shifts in all signals derived from the 3-hydroxy-3-methylbut-1-enyl side-chain and in the olefinic coupling constant (J = 13 Hz; cf. J = 16 Hz) were apparent. As the coupling constant of 13 Hz cannot definitely be assigned to either a *cis* or *trans* orientated olefine [5] this new coumarin could, theoretically, have been any one of twenty-three possible isomers of *trans*-avicennol.

Comparison of the $Eu(fod)_3$-induced shifts [1, 6] in the PMR spectrum of the *O*-TMSi derivative of the new

Table 1. Comparison of chemical shifts (δ) of the protons of *trans*-avicennol (1) and the 'yellow oil'*

	3-H	4-H	3'-H	4'-H	2'-Me_2	OMe	1''-H	2''-H	3''-Me_2	OH
trans-Avicennol	6.27	8.06	5.69	6.65	1.48	3.80	6.95	6.81	1.48	2.58
J (Hz)	10	10	10	10			16	16		
'Yellow oil'	6.29	8.05	5.69	6.65	1.49	3.85	6.25	6.01	1.31	2.85
J (Hz)	10	10	10	10			13	13		

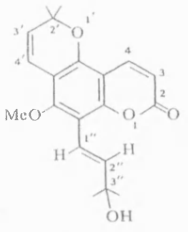
* The non-systematic numbering system adopted (see formula 1) is used to allow comparison with other coumarin shift data [1, 6].

Table 2. Comparison of induced shift values (relative to 3H = 1.00) for *trans*-avicennol and 'yellow oil' *O*-TMSi ethers with Eu(fod)$_3$*

	3-H	4-H	3'-H	4'-H	2'-Me$_2$	OMe	1''-H	2''-H	3''-Me$_2$	*O*-TMSi
trans-Avicennol	1.00	0.31	0.08	0.13	0.08	0.15	0.40	0.52	0.13	0.11
'Yellow oil'	1.00	0.32	0.09	0.15	0.08	0.16	0.32	0.25	0.28	0.19

* See Table 1.

compound with those of *O*-TMSi-*trans*-avicennol (Table 2) aided the structure assignment. No significant change was noted in shift ratios for protons of the lactone ring, pyran ring or OMe group. On the other hand the olefinic protons of the side-chain of the new compound were less shifted than in *trans*-avicennol but were once again of such a magnitude as to restrict side-chain attachment to C-8 of the coumarin nucleus [1]. The shifts of the *gem*-dimethyl and *O*-TMSi groups were, however, markedly larger than those of the corresponding *trans* side-chain indicating that these groups were closer to the point of complexation. These results are in agreement with those anticipated for the *cis* isomer of *O*-TMSi-avicennol (**2b**).



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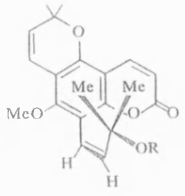
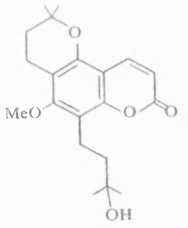


2a R = H
2b R = Si(Me)$_3$



3

The substitution pattern of the new coumarin was confirmed by hydrogenation to give the corresponding tetrahydro- derivative which proved identical to the previously synthesised tetrahydroavicennol (**3**) [1], thereby indicating that it was *cis*-avicennol (**2a**).

It has recently come to our notice that synthetically derived 7-methoxy-8-(3-hydroxy-3-methyl-*cis*-but-1-enyl) coumarin showed a high coupling constant (J = 12 Hz) for the side-chain olefinic protons and, like *cis*-avicennol, proved impossible to crystallise [7].

EXPERIMENTAL

Plant Material. Root bark of *Zanthoxylum elephantiasis* Macfad. was collected at Falmouth, Trelawny, Jamaica (Voucher: A.I. Gray 3) and at Zalaya, Santo Domingo, Dominican Republic (voucher: J. Jimenez 5972). Vouchers have been deposited at the herbarium of the Royal Botanic Garden, Edinburgh.

Extraction and isolation. The milled bark (800 g from A.I. Gray 3 collection) was extracted with petrol bp 40–60°. The conc petrol extract, after deposition of canthin-6-one, xanthoxyletin and avicennol, was subjected to PLC (1 mm) over Si gel. Elution with EtOAc–hexane (3:2) yielded dihydrocelerythrine, avicennin, dipetalolactone and triacontanoic acid [3]. On concn of the ethanolic supernatant from the recrystallisation of triacontanoic acid a yellow oil (**2a**, 95 mg, R_f 0.55 in PLC system) was obtained.

Cis-*avicennol* (**2a**). The yellow oil could not be obtained in crystalline form. Found M$^+$ 342.1453; $C_{20}H_{22}O_5$ requires 342.1467. UV λ_{max}^{EtOH} nm: 236, 275, 294. IR ν_{max}^{nujol} cm^{-1}: 3490, 1725, 1140. PMR (60 MHz, $CDCl_3$, δ): 1.31 (6H, *s*, 3''-Me$_2$), 1.49 (6H, *s*, 2'-Me$_2$), 2.85 (1H, *br s*, lost on D_2O exchange, 3''-OH), 3.85 (3H, *s*, OMe), 5.69, 6.65 (2H, AB*q*, J = 10 Hz, 3'-H and 4'-H), 6.25, 6.01 (2H, AB*q*, J = 13 Hz, 1''-H and 2''-H), 6.29, 8.05 (2H, AB*q*, J = 10 Hz, 3-H and 4-H). MS *m/e* (rel. int.): 342 (42%, M$^+$), 327 (100), 324 (19), 309(20).

Cis-*avicennol*-O-*TMSi-ether* (**2b**). **2a** (50 mg) was dissolved in dry C_6H_6 and an excess of BSA added. After 7 hr the mixture was evap. to dryness and the residue chromatographed on Si gel. Elution with EtOAc–hexane (1:1) gave a yellow oil (45 mg, R_f 0.71). UV λ_{max}^{EtOH} nm: 238, 275, 298. PMR (60 MHz, $CDCl_3$, δ): −0.24 (9H, *s*, $SiMe_3$), 1.38 (6H, *s*, 3''-Me$_2$), 1.47 (6H, *s*, 2'-Me$_2$), 3.81 (3H, *s*, OMe), 5.60, 6.59 (2H, AB*q*, J = 10 Hz, 3'-H and 4'-H), 5.78, 6.01 (2H, AB*q*, J = 13 Hz, 2''-H and 1''-H), 6.20, 8.00 (2H, AB*q*, J = 10 Hz, 3-H and 4-H).

Tetrahydroavicennol (**3**). **2a** (20 mg) in EtOH was hydrogenated over Adams catalyst. The reaction mixture was filtered and evap. to dryness. TLC [1] indicated a single product, R_f 0.4. The residue on recryst. from MeOH as colourless prisms (18 mg) mp 165–165.5°, identical in all respects (UV, IR, PMR, TLC, mmp) with an authentic sample of tetrahydroavicennol [1].

Cis-*avicennol in* Z. elephantiasis—*Jimenez 5972.* A similar extraction and isolation procedure indicated the presence of small amounts of *cis*-avicennol in this material also.

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REFERENCES

1. Gray, A. I., Waigh, R. D. and Waterman, P. G. (1975) *J. Chem. Soc., Perkin Trans.* **1**, 488.
2. Fish, F., Gray, A. I. and Waterman, P. G. (1975) *Phytochemistry* **14**, 2073.
3. Fish, F., Gray, A. I. and Waterman, P. G. (1976) *J. Pharm. Pharmac.* **28**, 69P.
4. Murray, R. D. H. and Forbes, I. T. (1976) *Tetrahedron Letters* 953.
5. Laszlo, P. and Schleyer, P. R. (1964) *Bull. Soc. Chim. Franc.* 87.
6. Gray, A. I., Waigh, R. D. and Waterman, P. G. (1974) *J. Chem. Soc., Chem. Comm.* 632.
7. Murray, R. D. H. and Forbes, I. T. (1976) unpublished results.

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Interactions of Coumarins with a Lanthanide Shift Reagent: Determination of Substitution Pattern

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The effects of $Eu(fod)_3$ on the ^1H n.m.r. spectra of 22 coumarins with alkyl, alkoxy, furano, and pyrano substituents and of coumarin itself have been studied, and a method for obtaining information on the substitution pattern of the coumarins has been developed.

THE frequent occurrence and structural diversity of natural coumarins [1] has led to the application of a variety of n.m.r. techniques to their structure elucidation, particularly the use of chemical shifts and coupling,[2] the internal nuclear Overhauser effect,[3,4] and recently ^{13}C n.m.r.[5-7] Some of these techniques are not readily available, and even when they are, the unambiguous structure determination of certain types of coumarin still presents problems.

We have described briefly [8] a simple, rapid, non-degradative, non-empirical method for the structure elucidation of coumarins using the n.m.r. shift reagent $Eu(fod)_3$. We explain here how it can be used as a practical method, when the method is most useful, and also where difficulties may be encountered.

To evaluate the approach fully we have used a variety of natural and synthetic coumarins (1)—(23) with different functional groups and substitution patterns.

METHOD

A full discussion of the use of lanthanide shift reagents (l.s.r.) in n.m.r. spectroscopy has been published [9] and an earlier review may also be found helpful [10]

It is essential that the solvent should not complex with the shift reagent, which reduces the choice, but we have found $CDCl_3$ ideal for coumarins when using $Eu(fod)_3$, and have had no reason to consider alternatives. For use in a standard 60 MHz spectrometer, we have dissolved coumarin (*ca.* 60 mg) in $CDCl_3$ (0.3 ml) containing tetramethylsilane as internal standard. The normal spectrum was obtained, and then $Eu(fod)_3$ (*ca.* 10 mg) added, dissolved, and the spectrum re-run. In each case the shifts of all the protons were measured relative to tetramethylsilane, so that the lanthanide-induced shift (l.i.s.) could be obtained. Thus, $\delta H_{[Eu(fod)_3]} - \delta H_{(untreated)} = l.i.s.$ In our approach the l.i.s. for each proton was then divided by the l.i.s. for the proton attached to C-3 of the coumarin. This gave a series of shift ratios which are independent of the weight of coumarin or shift reagent, and can be used to determine the substitution pattern of the coumarin.

We chose to use several additions (usually four) of shift reagent, and to plot the induced shift against the integral of the main resonance of the shift reagent, so as to obtain a series of straight lines which can be used to determine the shift ratios. This approach has advantages where there is overlapping of peaks, either in the original spectrum or after addition of $Eu(fod)_3$, when the precise position of a peak may be difficult to determine. In simple cases there is no advantage in the graphical approach, although more than one addition of $Eu(fod)_3$ is advisable, so that average shift values may be obtained as a precaution against random error.

Coumarins normally give very sharp resonances with good resolution, but addition of large amounts of shift reagent almost always broadens the lines. Thus there may be an advantage in using smaller amounts of coumarin initially, where instrument performance permits, so that less l.s.r. is needed. Concentrated solutions of the coumarin should be avoided for this reason.

Having obtained a series of shift ratios by either approach, there are two ways in which the data can be used. As a quick method, we have calculated (Tables 2—4) the shift ratios expected using average † complexation parameters for H, CH_3, pyran, and furan substituents in all positions (except, of course, C-3). Thus a comparison of the experimental ratios with those in Tables 2—4 may often give valuable structural information, sometimes amounting to total structure elucidation.

If it is considered desirable to refine the calculated figures for the specific coumarin under study, as we have done,[4,11,12] it is necessary to adjust ϕ and d (Figure 1) for an optimum fit to the experimental data.

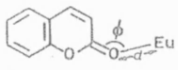
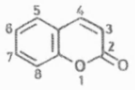


FIGURE 1

In our experience the easiest way to do this is to use a field map [13] and a Dreiding stereomodel. With the model flat on the map the carbonyl oxygen is placed on the mid-line of the field map. Movement along this line varies d, and rotation of the model in the plane of the map with the carbonyl oxygen always on the mid-line, varies ϕ. Rapid

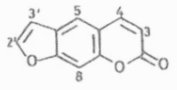
† Three coumarins (21)—(23) were excluded from the averaging (see Discussion section).

[1] B. E. Nielsen, *Dansk. Tidsskr. Farm.*, 1970, **44**, 111; R. D. H. Murray, *Aromatic Heteroaromatic Chem.*, 1976, **4**, 422.
[2] W. Steck and M. Mazurek, *Lloydia*, 1972, **35**, 418.
[3] T. Tomimatsu, M. Hashimoto, T. Shingu, and K. Tori, *Chem. Comm.*, 1969, 168; *Tetrahedron*, 1972, **28**, 2003.
[4] A. I. Gray, R. D. Waigh, and P. G. Waterman, *J.C.S. Perkin I*, 1976, 488.
[5] Ching-jer Chang, H. G. Floss, and W. Steck, *J. Org. Chem.*, 1977, **42**, 1337, and references cited.
[6] K. K. Chan, D. D. Giannini, A. H. Cain, J. D. Roberts, W. Porter, and W. F. Trager, *Tetrahedron*, 1977, **33**, 899.
[7] D. Bergenthal, K. Szendrei, and J. Reisch, *Arch. Pharm.* 1977, **310**, 390.
[8] A. I. Gray, R. D. Waigh, and P. G. Waterman, *J.C.S. Chem. Comm.*, 1974, 632.
[9] A. F. Cockerill, G. L. O. Davies, R. C. Harden, and D. M. Rackham, *Chem. Rev.*, 1973, **73**, 553.
[10] P. V. DeMarco, *Lloydia*, 1972, **35**, 362.
[11] F. Fish, A. I. Gray, R. D. Waigh, and P. G. Waterman, *Phytochemistry*, 1976, **15**, 313.
[12] A. I. Gray, R. D. Waigh, and P. G. Waterman, *Phytochemistry*, 1977, **16**, 1017.
[13] R. M. Wing, T. A. Early, and J. J. Uebel, *Tetrahedron Letters*, 1972, 4153.



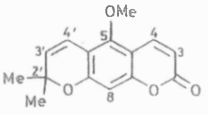
Substituent

	3	4	5	6	7	8
(1)	H	H	H	H	H	H
(2)	H	H	H	H	OMe	H
(3)	H	H	H	Me	H	H
(4)	H	Me	H	H	OMe	H
(5)	H	Me	H	OMe	H	H
(6)	H	H	OMe	H	OMe	H
(7)	H	H	H	OMe	OMe	H
(8)	H	Me	H	H	OMe	Me



Substituent

	5	8
(9)	H	OMe
(10)	OMe	H
(11)	H	$OCH_2CH{=}C(Me)_2$



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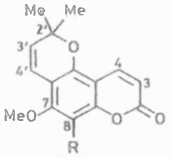
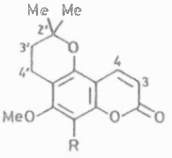
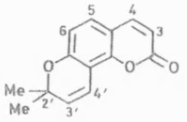




(13) R = *trans*-CH=CH·C(OSiMe$_3$)Me$_2$
(14) R = *cis*-CH=CH·C(OSiMe$_3$)Me$_2$
(15) R = *trans*-CH=CH·C(OH)Me$_2$
(16) R = *cis*-CH=CH·C(OH)Me$_2$
(17) R = *trans*-CH=CH·C(Me)=CH$_2$
(20) R = CH_2·CH=CMe$_2$

(18) R = $CH_2CH_2C(H)Me_2$
(19) R = $CH_2CH_2C(OH)Me_2$



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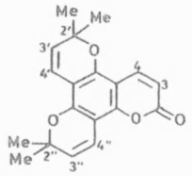

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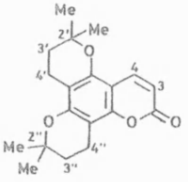


(23)

visual inspection of proton positions on the map will then reveal if the shift ratios are reasonable for the compound in question. When a ' best fit ' has been obtained, the proton positions can be marked on the field map so that θ and R can be measured for each one (Figure 2).

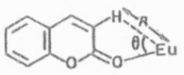


FIGURE 2

The value of $(3\cos^2\theta - 1)/R^3$ can then be calculated for each proton to give the relative shifts. For comparison with the experimental figures, the relative shifts must be normalised to relative shift 3-H ≡ 1.00, and this is done by dividing each relative shift figure by the value of $(3\cos^2\theta - 1)/R^3$ obtained for 3-H. To save time on the calculations we plotted $3\cos^2\theta - 1$ for all possible values of θ, so that values did not need to be calculated individually.

Where substituents are not in the plane of the ring, as with methylpyran and methoxy (see Discussion section) measurements of θ and R are more awkward, but once ϕ and d have been chosen with substituents which do fall in the plane of the field map the problem is simplified.

The experimental and calculated values obtained as above for all the coumarins are given in Table 1.*

* Non-systematic numbering has been used wherever necessary to facilitate comparisons between simple and polycyclic coumarins.

DISCUSSION

Normally correlations of structure with l.i.s. are three-dimensional problems, for which a computer is required to calculate the direction and length of the lanthanide–ligand bond which give a ' best fit ' to the observed data. The advantage of the present method is that the problem of location of the europium atom has been reduced to two dimensions so that a computer is not necessary. While this involves assumptions about the bonding of coumarins to $Eu(fod)_3$ which may not be universally valid, a good fit has been obtained for a large number of coumarins for all substituents (Table 1), and the method has been applied successfully.[4,11,12,14] Theoretical calculations of the electron densities in coumarins also tend to support our findings.[15]

The only examples in which a poorer than usual fit was obtained were those with 7,8-pyran substitution [seselin (21), dipetalolactone (22), and tetrahydrodipetalolactone (23)]. For these three compounds the 4-H shift was less than calculated if the ϕ and d values were altered to give a good fit elsewhere, and the ϕ and d values thus obtained were atypical (Table 1). Possibly a better fit could be obtained by allowing movement of the europium atom or the ring substituents out of the plane of the coumarin ring or to allow the ring to become non-planar, but the present method could not then be used.

Some coumarins [*e.g.* (7), (15), (16), (19)] possess additional complexation sites for $Eu(fod)_3$. Obviously the presence of such a site destroys the simple relationship between induced shift and substituent position which is the basis of the method. Where the additional site is a side-chain hydroxy, as in *cis*- and *trans*-avicennol,[4,12] it can be conveniently protected as the trimethylsilyl ether. In other cases the method of protection would have to be devised specifically, but a special problem may be posed by the presence of *o*-methoxys. An isolated methoxy does not complex significantly with $Eu(fod)_3$, but *o*-methoxys do,[9] and the difference has been used to confirm the structure of 2,4,5-trimethoxystyrene.[16] Similar complexation occurs with the methoxys of scoparon (7) and it is clear from the figures in Table 1 that the relative l.i.s. for all protons in scoparon (except by definition 3-H) are much larger than usual. Subtraction of average values for shifts in each position from Table 2, calculated for complexation at the lactone carbonyl only, gives an

TABLE 1

Observed and calculated shift values for coumarins, relative to 3-H ≡ 1.00 (calculated values in parentheses)

			Substituent position							
Compound	ϕ(°)	d/Å	4	5	6	7	8	2′	3′	4′
Coumarin (1)	156	3.00	0.31(0.32)	0.19(0.19) [a]	0.15(0.14) [a]	0.19(0.17) [a]	0.31(0.33)			
Herniarin (2)	159	3.30	0.34(0.34)	0.20(0.20)	0.16(0.15)	0.10(0.12)	0.29(0.32)			
6-Methylcoumarin (3)	158	3.25	0.29(0.32)	0.20(0.18)	0.10(0.11)	0.15(0.16)	0.27(0.31)			
4-Methylherniarin (4)	160	2.60	0.22(0.20)	0.22(0.20)	0.18(0.15)	0.12(0.12)	0.34(0.34)			
6-Methoxy-4-methylcoumarin (5)	160	2.60	0.20(0.20)	0.20(0.20)	0.11(0.11)	0.18(0.17)	0.33(0.34)			
Limettin (6)	158	3.25	0.31(0.32)	0.09(0.10) [c]	0.16(0.14)	0.13(0.14) [c]	0.28(0.31)			
Scoparon (7)	*b*		0.40	0.54	0.36	0.36	0.61			
4,8-Dimethylherniarin (8)	160	2.63	0.22(0.20)	0.22(0.20)	0.18(0.15)	0.13(0.12)	0.30(0.29)			
Xanthotoxin (9)	145	2.75	0.25(0.26)	0.20(0.18)			0.29(0.29)	0.10(0.10)	0.11(0.10)	
Bergapten (10)	158	3.45	0.34(0.33)	0.14(0.13)			0.30(0.32)	0.11(0.09)	0.12(0.11)	
Imperatorin (11)	142.5	2.50	0.31(0.30)	0.22(0.22)			*d*	0.12(0.12)	0.14(0.13)	
Xanthoxyletin (12)	159.5	3.35	0.30(0.33)	0.17(0.14)			0.30(0.32)	0.07(0.09)	0.05(0.08)	0.11(0.12)
trans-Avicennoltrimethylsilyl ether (13)	152.5	2.75	0.31(0.31)			0.15(0.15)	*d*	0.08(0.08)	0.08(0.08)	0.13(0.12)
cis-Avicennoltrimethylsilyl ether (14)	152.5	2.75	0.32(0.31)			0.16(0.15)	*d*	0.08(0.08)	0.09(0.08)	0.15(0.12)
trans-Avicennol (15)	*b*		0.40			0.40	*d*	0.14	0.14	0.26
cis-Avicennol (16)	*b*		0.35			0.29	*d*	0.09	0.08	0.18
Avicennin (17)	151	2.80	0.29(0.30)			0.13(0.13)	*d*	0.08(0.08)	0.08(0.08)	0.12(0.11)
Hexahydroavicennin (18)	151	2.80	0.30(0.30)			0.13(0.13)	*d*	0.09(0.08)	0.11(0.09)	0.13(0.12)
Tetrahydroavicennol (19)	*b*		0.30			0.60	*d*	0.12	0.20	0.41
Dipetaline (20)	152.5	2.75	0.31(0.31)			0.15(0.15)	*d*	0.08(0.08)	0.08(0.08)	0.12(0.11)
Seselin (21)	184	2.40	0.30(0.37)	0.20(0.21)	0.13(0.14)			0.05(0.06)	0.04(0.05)	0.23(0.23)
			4	2′	3′	4′	2″	3″	4″	
Dipetalolactone (22)	184	2.40	0.31(0.37)	0.08(0.10)	0.07(0.08)	0.09(0.10)	0.05(0.06)	0.03(0.05)	0.22(0.23)	
Tetrahydrodipetalolactone (23)	184	2.40	0.29(0.37)	0.07(0.11)	0.07(0.11)	0.08(0.11)	0.04(0.07)	0.05(0.07)	0.19(0.24)	

[a] Assignments uncertain owing to complex splitting and overlapping. [b] Two complexation sites. [c] See text. [d] See Table 5.

TABLE 2

Calculated shifts for simple substituents in all positions (θ 156°, d 3.0 Å), relative to 3-H ≡ 1.00

	Position				
Substituent	4	5	6	7	8
H	0.32	0.19	0.14	0.17	0.33
Me	0.19	0.15	0.11	0.13	0.29
OMe(C)	0.13	0.17	0.10	0.11	0.16
OMe(A)	0.16	0.11	0.10	0.15	0.50
OMe(O)	0.15	0.13	0.10	0.12	0.26

C = Clockwise, A = anticlockwise, O = out-of-plane (coumarin as drawn in this paper).

[14] K. K. Purushothaman, S. Vasanth, J. D. Connolly, and C. Labbé, *J.C.S. Perkin I*, 1976, 2594.

[15] C. Decoret and J. Royer, *Bull. Soc. chim. France*, 1976, 587; M. Abou-Asrali, C. Decoret, J. Royer, and J. Dreux, *Tetrahedron*, 1976, **32**, 1655.

[16] P. G. Waterman, *Phytochemistry*, 1976, **15**, 347.

TABLE 3

Calculated shifts for dimethylpyranocoumarins (ϕ 156°, d 3.0 Å), relative to 3-H ≡ 1.00

Ring fusion	Substituent 2′,2′-Me_2	3′-H	4′-H
6,5	0.08	0.08	0.16
5,6	0.09	0.08	0.11
7,6	0.09	0.08	0.11
6,7	0.09	0.09	0.14
8,7	0.16	0.11	0.12
7,8	0.10	0.13	0.33

TABLE 4

Calculated shifts for furanocoumarins (ϕ 156°, d 3.0 Å), relative to 3-H ≡ 1.00

Ring fusion	Substituent 2′-H	3′-H
6,5	0.09	0.15
5,6	0.09	0.11
7,6	0.09	0.10
6,7	0.09	0.13
8,7	0.13	0.12
7,8	0.13	0.31

approximate measure of the effect of *o*-methoxy complexation with scoparon (Figure 3). In a recent con-

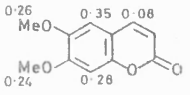
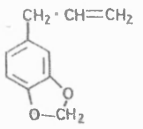


FIGURE 3

trasting example only simple complexation with a coumarin occurred,[14] despite the presence of 6,7-dimethoxy substitution. Clearly some further work needs to be done before the effect of molecular structure on methoxy complexation is fully understood. Complexation with *m*-methoxys does not appear to be significant, as shown by the results for limettin (6) (Table 1). No



(24)

methylenedioxy-substituted coumarins were readily available to us, but experiments with safrole (24) indicate that complexation between $Eu(fod)_3$ and the methylenedioxy group would be weak and unlikely to interfere with the method.

In most of our work we have assumed that methoxy substituents would adopt an out-of-plane conformation in order to minimise steric interactions with groups *ortho* to them. Where the *ortho*-substituents are small this may not be valid, results in other areas [17] suggesting that there is a favourable conformation for the methyl in the plane of the ring, stabilised by overlap of the lone pairs of the ether oxygen with the aromatic π-electrons. The good fit we have obtained in most cases suggests that the out-of-plane conformation is a reasonable approximation, except where a substituent on one side of the methoxy dictates a move towards the less hindered side. Thus in the case of limettin (6) the observed shift for what we assume to be the 5-methoxy is lower than calculated, unless rotation towards C-6 away from 4-H is invoked (Table 2). This may cause rotation of the 7-methoxy away from the 5-methoxy, resulting in a conformation which fits the observed shifts (Figure 4).

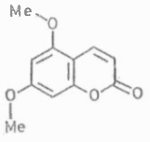


FIGURE 4

Clearly further work would be needed to justify these refinements, but there is a probability that low values may be observed for 5-methoxys in coumarins lacking 6-substitution, for this reason.

TABLE 5

Shifts for 8-isoprenyl substituents (from point of attachment to coumarin) [a]

Compound	Chemical shift (3-H ≡ 1.00)			
(11)	CH_2 0.29	CH 0.21	Me_2 0.13, 0.07	
(13)	CH 0.40(0.33)	CH 0.52(0.45)	$SiMe_2$ 0.11(0.11)	Me_2 0.13(0.12)
(14)	CH 0.32	CH 0.25	$SiMe_3$ 0.19	Me_2 0.28
(15)	CH 2.08	CH 2.56	OH 8.57	Me_2 1.59
(16)	CH 0.66	CH 0.74	OH 3.78	Me_2 0.81
(17)	CH 0.41(0.32)	CH 0.38(0.45)	Me 0.09(0.15)	$=CH_2$ 0.09, 0.03(0.15, 0.08)
(18)	CH_2 0.36	CH_2 0.24	CH [b]	Me_2 0.11
(19)	CH_2 2.92	CH_2 4.03	OH 7.35	Me_2 2.17
(20)	CH_2 0.35(0.32)	CH 0.54(0.48)	Me_2 0.08, 0.19(0.12, 0.15)	

[a] Calculated values, where given in parentheses, are for simple averages of in-plane conformations. [b] Not identified.

While the calculated figures in Table 2 refer to a limited number of substituents, it is clear that the figures for CH_3 and OCH_3 will serve as a reasonable approximation for hydrogens attached to the first carbon of longer chains. The flexibility of longer chains is such as only to allow estimates for the predicted shifts, but particularly where an 8-substituent is concerned shift differences are large enough to allow the positional assignment to be made with fair confidence.[4,11,12] In Table 5 we have given the observed and, in three cases, calculated values for flexible prenyl-derived side-chains in all the compounds we have had

[17] A. Hofer, *Tetrahedron Letters*, 1975, 3415.

the opportunity to evaluate. Where given, the calculated values are based on average values for two extreme conformations, so that only an approximate fit would be expected. In some cases extra information can be obtained from simplification of splitting patterns in the side chain, and even configurational information may be obtained.[12]

The relatively poor fit obtained for 7,8-pyranocoumarins may be tentatively explained by means of steric interactions between a relatively inflexible 8-substituent and the lactone ring oxygen. For such compounds the method still offers useful structural information on a more empirical basis, since the shift for the 8-substituent is larger than would be obtained elsewhere. Further work is in progress in an attempt to clarify this anomaly.

EXPERIMENTAL

Spectra were recorded on a Perkin-Elmer R12 n.m.r. spectrometer. Coumarins were obtained from a variety of natural and commercial sources, or by routine methylation of commercially available hydroxycoumarins. Structures were confirmed by comparison with literature data (u.v., i.r., n.m.r., m.s., m.p.) except for new compounds which were identified previously.[4,11,12]

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SECOIRIDOID AND TRITERPENIC ACIDS FROM THE STEMS OF *NAUCLEA DIDERRICHII*

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Key Word Index—*Nauclea diderrichii*; Rubiaceae; alkanes; alkanoic acids; triterpenic acids; secoiridoid; quinovic acid; 3-oxoquinovic acid; 3-*O*-glucosylquinovic acid; diderroside.

Abstract—The stem bark of *Nauclea diderrichii* has yielded diderroside, a new secoiridoid glucoside, as well as quinovic acid, 3-oxoquinovic acid and 3-*O*-glucosylquinovic acid. The hydrocarbon fraction was dominated by *n*-heptacosane and *n*-nonacosane, which accords with the predominance of *n*-octacosanoic acid in the alkanoic acid fraction.

INTRODUCTION

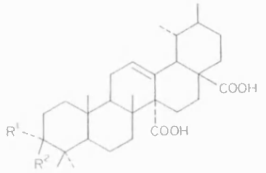
Nauclea diderrichii (De Wild) Merr. (*Sarcocephalus diderrichii* De Wild) is a large evergreen tree abundant in the rain forests of West Africa. The bark finds some local use in the treatment of gonorrhoea, for stomach pains, fever and sometimes diarrhoea. Its strength as a timber and resistance to termites make it valuable in construction work, although sawmill workers have been reported to suffer from cardiac poisoning associated with its use [1].

Previous work on *N. diderrichii* has yielded a number of alkaloids [2–7], some of which may be artefacts [8]. Our own results with the alkaloids will be reported separately. The non-alkaloidal constituents reported by other workers include naucleol, antiarol and the secoiridoid naucledal [7] and, somewhat earlier, 'sitosterol palmitate', *meso*-inositol and *O*-methyl-*meso*-inositol from the heartwood [9]. Our analysis of the sterol ester mixture from the stem bark has been reported previously [10].

RESULTS AND DISCUSSION

Extraction of the powdered bark with petrol, followed by CC, gave four fractions, identified as hydrocarbons, sterol esters [10], sterols and fatty acids. The sterols proved to be an unexceptional mixture of sitosterol, stigmasterol and campesterol. The hydrocarbons were mainly normal alkanes, the most abundant being *n*-heptacosane, followed by *n*-nonacosane. This pattern was reflected in the alkanoic acids, of which the most abundant was *n*-octacosanoic acid. The relative abundancies of the alkanes and alkanoic acids may be of some chemotaxonomic value since they are certainly easier to isolate and identify, using GC/MS, than more complex metabolites.

Chloroform extraction gave further amounts of the four types of mixture obtained from the petrol extract. More polar fractions yielded two triterpenic acids tentatively identified from mass spectral and NMR data as quinovic acid (**1a**) [11] and the rarely reported [12] 3-oxoquinovic acid (**1b**). These were separated by prep. TLC as their methyl esters, one of which was shown to be identical to a sample of quinovic acid obtained elsewhere, and similarly methylated. The latter sample also contained 3-oxoquinovic acid, which may indicate that it is more common than supposed. The 3-oxoquinovic acid dimethyl ester was converted to quinovic acid dimethyl ester on reduction with sodium borohydride by analogy with the reduction of 3-oxocadambagenic acid dimethyl ester described by previous workers [11]. An even more polar fraction from this extract was shown to contain quinovic acid 3-*O*-glucoside (**1c**), purified and characterized as the dimethyl ester tetra-acetate. This glucoside was first reported from the Rubiaceae [13].



1a $R^1 = OH$, $R^2 = H$
1b $R^1 = R^2 = O$
1c $R^1 = Oglu$, $R^2 = H$

The water-soluble part of the chloroform extract contained small quantities of a substance later designated WF2A, larger amounts of which were present in the methanol extract. The water-soluble portion of the latter was subjected to charcoal-CC to remove sugars, and then gave a fraction which HPLC showed to contain a complex mixture. One component of the mixture was separated as a pure compound by semi-prep. HPLC, and called WF2A, identical to the smaller amount of material isolated from the chloroform extract. Experience showed WF2A to possess limited stability in solution, but sufficient data were obtained on samples soon after purification to allow structure elucidation.

The UV spectrum of WF2A showed absorption at 234 nm, typical of the O–C=C–COOMe system of iridoids [14]. This suggestion was supported by the ^{1}H NMR spectrum which showed a one-proton singlet at δ7.55 (H-3) and a three-proton singlet at 3.71 (COOMe-4). A three-proton singlet at 2.07 suggested an acetyl

group, and a three-proton doublet ($J = 7$ Hz) at 1.40 correlated well with a methyl group with a hydroxyl or acetoxy group on the neighbouring carbon atom. Other signals provisionally assigned were a doublet ($J = 8$ Hz) at 5.80 (H-1) and a one-proton multiplet at 5.25 (H-8) which suggested that an acetoxy group was attached at C-8 as well as the C-10 methyl. There was a doublet at 4.80 ($J = 7$ Hz) for the anomeric proton of a sugar, for the remaining protons of which there was complex absorption in the region 3.3–4.08. Other separated resonances assigned subsequently were a two-proton doublet ($J = 7$ Hz) at 2.33 and a multiplet for one proton at 2.20.

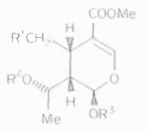
Hydrolysis with β-glucosidase showed that the sugar was β-D-glucose, although we could not isolate the aglucone, and the presence of a β-glucoside was confirmed by the ^{13}C NMR spectrum, which showed the characteristic resonances [15] at 99.7, 77.2, 76.7, 73.7, 70.8 and 61.9. Other resonances confirmed the iridoid skeleton [14], with C-1 at 97.2, C-3 at 154.2, C-4 at 110.4, C-11 at 169.6, methoxy at 52.6 and C-10 methyl at 19.1. The acetoxy group was confirmed by resonances at 21.8 and 174.0. Other signals, consistent with a secoiridoid structure and subsequently assigned, were located at 70.5 (C-8), 43.6 (C-9) and 29.6 (C-5). A broadened signal at 36.4, a triplet in the off-resonance spectrum, was assigned to C-6 which left C-7 unaccounted for.

At this stage we were able to make a tentative structure assignment as **2a**, excluding C-7, and sought further evidence from mass spectrometry. However, even direct probe insertion with chemical ionization failed to give a molecular ion. This problem was overcome with field desorption, which indicated a MW of 464, and this was confirmed by desorption–chemical ionization [16], the latter technique giving the simplest spectrum. With a consideration of the previous data, the missing carbon had to be part of a carboxylic acid group, so that in the ^{13}C NMR spectrum it may have been superimposed on one of the other carbonyl carbons; shortage of material has prevented confirmation of this by changing solvent. The broadening of the C-6 signal may also have been reflected in broadening of the C-7 signal, if the former was attributable to conformational changes caused by alternate hydrogen bonding to ester groups on either side, and may have obscured the presence of the C-7 signal.

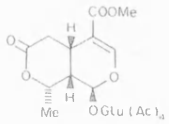
It was apparent that WF2A was a secoiridoid, previously unknown, to which we gave the name 'diderroside', and allocated structure **2a**. Acetylation gave diderroside tetra-acetate (**2b**), previously known as a synthetic intermediate [17], for which the ^1H NMR spectrum was consistent with the proposed structure. Methylation of the tetra-acetate with diazomethane confirmed the presence of the free carboxylic acid group, with a second methoxy signal in the ^1H NMR spectrum, and gave a product (**2c**) with sufficient volatility to allow accurate mass measurement of the molecular ion ($C_{26}H_{35}O_{15}$) and several fragments.

As final confirmation of the structure a series of decoupling experiments was carried out on methyl diderroside tetra-acetate (**2c**) at 300 MHz, which allowed assignment of all the resonances.

The question of the relative stereochemistry of diderroside (**2a**) was fortunately solved by the very recent structure elucidation [18] of alpigenoside (**2d**), for which the stereochemistry has been defined by conversion to tetra-acetylkingiside (**3**), of known absolute configuration [17]. Comparison of diderroside tetra-acetate methyl ester with penta-acetylalpigenoside by TLC and 300 MHz NMR showed that diderroside is des-7-*O*-methyl-8-*O*-acetylalpigenoside, with all four secoiridoid asymmetric centres in the same relative configurations. Shortage of material prevented measurement of the optical rotation of methyldiderroside tetra-acetate which, by comparison with the measured rotation of penta-acetylalpigenoside [18], would confirm the absolute stereochemistry. However, it is highly improbable that diderroside has a different absolute stereochemistry from alpigenoside.



2a R^1 = COOH, R^2 = MeCO, R^3 = Glu
2b R^1 = COOH, R^2 = MeCO, R^3 = Glu (Ac)$_4$
2c R^1 = COOMe, R^2 = MeCO, R^3 = Glu (Ac)$_4$
2d R^1 = COOMe, R^2 = H, R^3 = Glu



3

EXPERIMENTAL

Plant material was collected from a forest reserve on the Benin–Ijebu-Ode road, Nigeria and identified by Mr. Daramola, senior plant collector of the Forestry Research Institute of Nigeria. This identification was confirmed by comparison with voucher specimens at the institute's herbarium. Herbarium specimens have been deposited with the University of Manchester Museum.

Extraction and separation. The powdered stem bark (4 kg) was extracted with cold petrol (bp 40–60°, 10 L, 2 × 5 L) for 48 hr each time. The combined residue (9.13 g) was extracted with $CHCl_3$ to give a sticky residue (33 g). The MeOH extract of the stem bark was a brown powder (370 g). The powdered heartwood (4 kg) was extracted only with cold MeOH (15 L, 3 × 6 L) to give 217 g of a brown powder.

The petrol extract (6 g) was chromatographed on a Si gel column, eluting with petrol (bp 60–80°) with increasing proportions of $CHCl_3$ to yield an alkane fraction (1.70 g), steryl esters (0.42 g), sterols (2.00 g) and fatty acids (0.33 g). The $CHCl_3$ extract (20 g) was extracted with petrol to give 7.25 g of material similar to the petrol extract. The petrol insoluble material was further divided into H_2O soluble (1.54 g) and H_2O insoluble (7.85 g) material. The H_2O-insoluble residue (2 g) was chromatographed on Si gel using petrol, petrol–$CHCl_3$ and $CHCl_3$–MeOH to give (with 5% MeOH in $CHCl_3$) a mixture of quinovic acid and 3-oxoquinovic acid (340 mg). Elution with 10% MeOH in $CHCl_3$ gave 3-*O*-glucosyl-quinovic acid (90 mg).

The MeOH extract (250 g) was extracted with H_2O to give a brown gum (110 g). This material (25 g) was chromatographed on a prepared charcoal column, eluting first with H_2O and then with aq. MeOH (5–40%). Fractions obtained with 20–30% MeOH were bulked and evaporated at 30–40° *in vacuo* to give a yellow powder (1.52 g) which gave negative tests for reducing sugars. This powder was dissolved in 10% MeCN and chromatographed in 100 µl aliquots on a semi-prep. HPLC column, eluting with 10% MeCN and detecting at 254 nm. Fractions containing the major component were combined and freeze-dried to give a light off-white powder (46 mg) which undoubtedly represented only a small part of the material originally present, but was pure by analytical HPLC.

The only non-alkaloidal component isolated from the heartwood was *O*-methylinositol, which has previously been reported from this species [9].

Hydrocarbons. GC/MS using a 1% Dexsil 300 column, temp. programmed at 100° for 2 min followed by a rise of 8°/min to 250° gave the following percentages (chain lengths): *n*-alkanes 0.4 (14), 1.0 (15), 2.9 (16), 5.0 (17), 4.2 (18), 1.7 (19), 2.8 (20), 2.5 (21), 2.7 (22), 3.1 (23), 2.7 (24), 7.5 (25), 2.9 (26), 30.5 (27), 4.3 (28), 13.7 (29), 7.0 (30), 3.5 (31), 1.8 (32); *n*-alkenes C_{17}, C_{27}, *iso*-alkanes C_{17}–C_{25}, none greater than 0.3%.

Alkanoic acids. These showed the expected carbonyl absorption at 1705 cm^{-1} and ^{1}H NMR absorption at δ9.8 (*br s*, COOH), 1.25 [*br s*, $(CH_2)_n$] and 0.9 (*t*, Me). Methylation with BF_3–MeOH complex gave the methyl esters, with IR absorption at 1748 cm^{-1} and an additional ^{1}H NMR spectral peak at δ3.65 (OMe). GC/MS using a 1% Dexsil 300 column programmed at 150° for 2 min and a rise of 10°/min to 270° gave the following percentages (chain lengths): 10.1 (16), 13.6 (18, A1), 9.0 (18), 3.2 (24), 16.0 (26), 2.1 (27), 39.8 (28), 6.0 (30).

Quinovic acid and 3-oxo-quinovic acid. The mixture of these two components obtained by chromatography had mp 267° (decomp.) and showed IR carbonyl absorption at 1695 cm^{-1}; ^{1}H NMR (80 MHz, DMSO-d_6) δ12.1 (*br*, COOH), 5.5 (*br s*, H-12), 3.3 (*br m*, H-3), 0.8–0.9 (*m*, Me). Separation of the acids was not feasible, so the mixed acids were methylated with CH_2N_2 and separated by prep. TLC on Si gel, developing with $CHCl_3$–C_6H_6 (9:1). From 100 mg of crude mixed acids was obtained 27 mg dimethyl quinovate and 40 mg dimethyl 3-oxoquinovate. Each was crystallized from petrol (bp 40–60°) and then from petrol containing a small amount of MeOH. Dimethyl quinovate had mp 163–165° (lit. [19–21] 169–171°, 179.5–181°, 174–176°) $[\alpha]_D$ +73.3° ($CHCl_3$, *c* 1.76), IR ν_{max} cm^{-1}: 3650, 1720, 1700; ^{1}H NMR (300 MHz, $CDCl_3$): δ5.66 (1H, *t*, *J* = 2 Hz, H-12), 3.62 (3H, *s*, COOMe), 3.61 (3H, *s*, COOMe), 3.19 (1H, *q*, *J* = 6 Hz, H-3), 0.91 (3H, *d*, Me), 0.87, 0.85, 0.83, 0.74 (12H, 4*s*, 4 × Me), 0.82 (3H, *d*, Me); EIMS 70 eV, *m/z* (rel. int.): 514.3624 (13.7), calc. for $C_{32}H_{50}O_5$, 514.3645 [M]$^+$, 496.3442 (2.6), calc. for $C_{32}H_{48}O_4$, 496.3552 [M – H_2O]$^+$, 395.3402 (6.0), calc. for $C_{28}H_{43}O$, 395.3314 [M – 2 × COOMe – H]$^+$, 306.1810 (26.7), calc. for $C_{18}H_{26}O_4$, 306.1831 [M – $C_{14}H_{24}O$]$^+$, 207.1748 (100), calc. for $C_{14}H_{23}O$, 207.1749 [M – $C_{18}H_{25}O_4$]$^+$, 190.1724 (74.2), calc. for $C_{14}H_{22}$, 190.1721 [M – H_2O – $C_{18}H_{24}O_4$]$^+$. Prominent ions not accurately mass measured were 482 [M – 32]$^+$ (24.3), 464 [M – 50]$^+$ (3.8), 455 [M – 59]$^+$ (17.2), 328 [M – 186]$^+$ (6.9), 274 [M – 240]$^+$ (62.8), 247 [M – 267]$^+$ (50.9). (Found: C, 74.2; H, 9.8. Calc. for $C_{32}H_{50}O_5$: C, 74.6; H, 9.8%.)

Dimethyl 3-oxoquinovate had mp 144–144.5° (lit. [12] 156–157°), $[\alpha]_D$ +98.0° ($CHCl_3$, *c* 1.00), IR ν_{max} cm^{-1}: 1720, 1690, ^{1}H NMR (300 MHz, $CDCl_3$): δ5.69 (1H, *t*, H-12), 3.64 (6H, *s*, 2 × OMe), 2.5 (2H, *m*, H-2), 0.83–1.04 (18H, 2*d*, 4*s*, 6 × Me). EIMS 70 eV, *m/z* (rel. int.): 512.3492 (27.4), calc. for $C_{32}H_{48}O_5$, 512.3502 [M]$^+$, 453.3368 (57.5), calc. for $C_{30}H_{45}O_3$, 453.3369 [M – COOMe]$^+$, 306.1834 (23.5), calc. for $C_{18}H_{26}O_4$, 306.1831 [M – 206]$^+$, 274.1565 (45.0), calc. for $C_{17}H_{22}O_3$, 274.1569 [M – 238]$^+$. Prominent ions not accurately mass measured were 480 [M – 32]$^+$ (67.4), 421 [M – 91]$^+$ (11.3), 393 [M – 129]$^+$ (25.9), 262 [M – 250]$^+$ (17.7), 247 [M – 265]$^+$ (35.3), 215 [M – 297]$^+$ (46.7), 205 [M – 307]$^+$ (38.9), 128 [M – 384]$^+$ (20.2), 105 [M – 407]$^+$ (33.1), 91 [M – 421]$^+$ (50.4), 55 [M – 457]$^+$ (62.3), 41 [M – 471]$^+$ (100). (Found: C, 75.1; H, 9.7. Calc. for $C_{32}H_{48}O_5$: C, 75.0; H, 9.4%.)

A sample of quinovic acid (see acknowledgements) was found, after methylation, to give two spots on TLC, corresponding to dimethyl quinovate and dimethyl 3-oxoquinovate, in three solvent systems.

Dimethyl 3-oxoquinovate was converted to dimethyl quinovate (mp, mmp, TLC) by reduction in MeOH with $NaBH_4$.

3-O-Glucosyl-quinovic acid. This compound was difficult to purify and characterize owing to poor solubility in most solvents and lack of mobility on TLC. The dimethyl ester tetra-acetate was obtained as a white powder after prep. TLC on Si gel using $CHCl_3$–MeOH (4:1), and had mp 117–120°, broad IR band at 1750 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ5.7 (1H, *m*, H-12), 5.6 (1H, *d*, *J* = 8 Hz, H-1'), 4.1–5.3 (5H, *m*, H-2', H-3', H-4', H-5' and H-6'), 3.65 (3H, *s*, COOMe), 3.63 (3H, *s*, COOMe), 2.0–2.14 (12H, 4*s*, 4 × COMe), 0.78–0.98 (18H, *m*, 6 × Me). EIMS 70 eV *m/z* (rel. int.): 496.3663 (1.5), calc. for $C_{32}H_{48}O_8$, 496.3552 [M – glucose tetra-acetate]$^+$, 331.1083 (8.7), calc. for $C_{14}H_{19}O_9$, 331.1029 [M – dimethyl quinovate]$^+$, 271.0890 (1.9), calc. for $C_{12}H_{15}O_7$, 271.0818 [M – dimethyl quinovate – MeCOOH]$^+$, 190.1746 (7.2) calc. for $C_{14}H_{22}$, 190.1721 [M – glucose tetra-acetate – $C_{18}H_{24}O_8$]$^+$, 169.0508 (24.7), calc. for $C_8H_9O_4$, 169.0501 [acetylated glucose fragment]$^+$. No molecular ion was observed; the base peak was at *m/z* 43. Acid hydrolysis gave glucose (identified by PC) and, after remethylation with CH_2N_2, dimethyl quinovate, identified by comparison with an authentic sample.

Diderroside. This compound was obtained as a white amorphous powder with no discrete mp. $[\alpha]_D$ –34.6° (MeOH; *c* 1.16), CD (MeOH; *c* 0.40) $\Delta\varepsilon_{220}$ –4.18, $\Delta\varepsilon_{249}$ +0.47, UV λ_{max}^{MeOH} nm (log ε): 234 (3.91), IR ν_{max}^{KCl} cm^{-1}: 3400, 1700. ^{1}H NMR (300 MHz, D_2O): δ7.55 (1H, *s*, H-3), 5.8 (1H, *d*, *J* = 8 Hz, H-1), 5.25 (1H, *m*, H-8), 4.8 (1H, *d*, *J* = 8 Hz, H-1'), 3.71 (3H, *s*, COOMe), 3.3–4.08 (7H, *m*, H-2', H-3', H-4', H-5', H-6a', H-6b', H-5), 2.33 (2H, *d*, *J* = 7 Hz, H-6), 2.2 (1H, *m*, H-9), 2.07 (3H, *s*, COMe), 1.4 (3H, *d*, *J* = 7 Hz, H-10). ^{13}C NMR (25.1 MHz, D_2O): δ174.0 (*s*, COMe), 169.6 (*s*, C-11), 154.2 (*d*, C-3), 110.4 (*s*, C-4), 99.7 (*d*, C-1'), 97.2 (*d*, C-1), 77.2 (*d*, C-5'), 76.7 (*d*, C-3'), 73.7 (*d*, C-2'), 70.8 (*d*, C-4'), 70.5 (*d*, C-8), 61.9 (*t*, C-6'), 52.6 (*q*, OMe), 43.6 (*d*, C-9), 36.4 (*t*, C-6), 29.6 (*d*, C-5), 21.8 (*q*, MeCO), 19.1 (*q*, C-10). FDMS *m/z* (rel. int.): 487 [M + Na]$^+$ (100), 469 [M – 18 + Na]$^+$ (12.5), 464 [M]$^+$ (10.0), 446 [M – 18]$^+$ (6.0), 437 [M – 27]$^+$ (7.2), 406 [M – 58]$^+$ (15.7). DCI (NH_3) *m/z* (rel. int.): 464 [M]$^+$ (4.0), 447 [M + H – H_2O]$^+$ (4.0), 422 [M + H – 43]$^+$ (3.7), 405 [M – 59]$^+$ (1.6), base peak 180 [glucose]$^+$. A small sample of diderroside was hydrolysed with β-glucosidase in aq. soln, freeze-dried and chromatographed on paper to confirm glucose as a hydrolysis product. A further small sample was acetylated with Ac_2O–pyridine and the product purified by prep. TLC on Si gel using $CHCl_3$–MeOH (4:1) and obtained as a gum; ^{1}H NMR (300 MHz, $CDCl_3$): δ7.42 (1H, *s*, H-3), 5.59 (1H, *d*, *J* = 6 Hz, H-1), 5.27 (1H, *t*, *J* = 9 Hz, H-3'), 5.15 (1H, *m*, H-8), 5.13 (1H, *t*, *J* = 9 Hz, H-4'), 5.01 (1H, *dd*, *J* = 9 Hz, H-2'), 4.99 (1H, *d*, *J* = 6 Hz, H-1'), 4.31 (1H, *dd*, *J* = 4.5, 12 Hz, H-6a'), 4.16 (1H, *d*, *J* = 12 Hz, H-6b'), 3.77 (1H, *m*, H-5'), 3.70 (3H, *s*, OMe), 3.3 (1H, *m*, H-9), 2.64 (1H, *m*, H-6a), 2.42 (1H, *m*, H-6b), 2.1 (1H, *m*, H-5), 1.99–2.08 (15H, 5*s*, 5 × COMe), 1.32 (3H, *d*, *J* = 6 Hz, H-10).

This sample of diderroside tetra-acetate was treated with ethereal CH_2N_2 to give the methyl ester as a gum; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.41 (1H, *s*, H-3), 5.57 (1H, *d*, J = 6 Hz, H-1), 5.24 (1H, *t*, J = 9 Hz, H-3'), 5.12 (1H, *t*, J = 9 Hz, H-4'), 5.1 (1H, *m*, H-8), 5.03 (1H, *t*, J = 9 Hz, H-2'), 4.95 (1H, *d*, J = 7.5 Hz, H-1'), 4.28 (1H, *dd*, J = 4.5, 12 Hz, H-6a'), 4.14 (1H, *dd*, J = 2, 12 Hz, H-6b'), 3.72 (1H, *m*, H-5'), 3.67 (3H, *s*, OMe), 3.62 (3H, *s*, OMe), 3.26 (1H, *m*, H-5), 2.56 (1H, *dd*, J = 6, 16.5 Hz, H-6a), 2.49 (1H, *dd*, J = 7.5, 16.5 Hz, H-6b), 2.14, 2.06, 2.01, 1.98, (15H, 5*s*, 5 × COMe), 2.1 (1H, *m*, H-9), 1.31 (3H, *d*, J = 6 Hz, H-10). These assignments were confirmed by decoupling H-5', H-5, H-9 and H-10. EIMS 70 eV, *m/z* (rel. int.): 587.1586 (0.1), calc. for $C_{26}H_{35}O_{15}$, 587.1583 $[M]^+$, 239.0913 (15.6), calc. for $C_{12}H_{15}O_5$; 239.0919 $[M - \text{glucose tetra-acetate} - MeCOOH]^+$, 165.0556 (73.0), calc. for $C_9H_9O_3$; 165.0554 [3-carbomethoxy-5-vinylpyrilium]$^+$, 139.0396 (26.4), calc. for $C_7H_7O_3$; 139.0395 [3-carbomethoxypyrilium]$^+$.

Comparison of methyl diderroside tetra-acetate with alpigenoside penta-acetate (300 MHz ^{1}H NMR, TLC) showed that the two were identical.

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REFERENCES

1. Henry, T. A. (1924) *Plant Alkaloids* p. 443. Churchill, London.
2. McLean, S. and Murray, D. G. (1970) *Can. J. Chem.* **48**, 867.
3. McLean, S. and Murray, D. G. (1972) *Can. J. Chem.* **50**, 1478.
4. Murray, D. G., Szakolcai, A. and McLean, S. (1972) *Can. J. Chem.* **50**, 1486.
5. Dmitrienko, G. I., Murray, D. G. and McLean, S. (1974) *Tetrahedron Letters* 1961.
6. Dmitrienko, G. I., Szakolcai, A. and McLean, S. (1974) *Tetrahedron Letters* 2599.
7. McLean, S. and Murray, D. G. (1972) *Can. J. Chem.* **50**, 1496.
8. Hemingway, S. R. and Phillipson, J. D. (1979) *Indole and Biogenetically Related Alkaloids* (Phillipson, J. D. and Zenk, M. H., eds.) p. 70. Academic Press, New York.
9. King, F. E. and Jurd, L. (1953) *J. Chem. Soc.* 1192.
10. Adeoye, A. O., Hemingway, S. R. and Waigh, R. D. (1981) *J. Pharm. Pharmacol.* **33**, 53P.
11. Sahu, N. P., Mahato, S. B., Banerji, N. and Chakravarti, R. N. (1974) *Indian J. Chem.* **12**, 284.
12. Hui, W. H. and Li, M. M. (1975) *Phytochemistry* **15**, 985.
13. Tschesche, R., Duphorn, I. and Snatzke, G. (1963) *Justus Liebig's Ann. Chem.* **667**, 151.
14. El-Naggar, L. J. and Beal, J. L. (1980) *J. Nat. Prod.* **43**, 649.
15. Bock, K., Jensen, S. R. and Nielsen, B. J. (1976) *Acta Chem. Scand. Ser. B* **30**, 743.
16. Hostettman, K., Doumas, J. and Hardy, M. (1981) *Helv. Chim. Acta.* **64**, 297.
17. Inouye, H., Yoshida, T., Tobita, S., Tanaka, K. and Nishioka, T. (1974) *Tetrahedron* **30**, 201.
18. Bailleul, F., Leveau, A. M. and Durand, M. (1981) *J. Nat. Prod.* **44**, 573.
19. Raffauf, R. F., Le Quesne, P. W. and Ghosh, P. C. (1978), *J. Nat. Prod.* **41**, 432.
20. Fujita, E., Fujita, T. and Suzuki, T. (1967) *Chem. Pharm. Bull.* **15**, 1682.
21. Ferrari, F., Kiyan de Cornelio, I., Delle Monache, F. and Marini Bettolo, G. B. (1981) *Planta Med.* **43**, 24.

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DESOXYCORDIFOLINIC ACID FROM *NAUCLEA DIDERRICHII*

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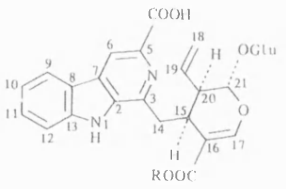
Key Word Index—*Nauclea diderrichii*; Rubiaceae; heartwood; alkaloids; β-carboline; desoxycordifolinic acid.

Abstract—The heartwood of the stems of *Nauclea diderrichii* yielded as its major alkaloid the parent diacid of desoxycordifoline, which has been named desoxycordifolinic acid.

INTRODUCTION

Previous work on the stems of *Nauclea diderrichii*, a large evergreen timber tree common in the rain forests of west tropical Africa, has shown the plant to be capable of elaboration of simple β-carbolines [1–6], in common with other species of the Rubiaceae, and a seco-iridoid [7]. A series of pyridines and β-carboline-pyridines has also been reported from the species [1–6], after extraction procedures which utilized ammonia; the presence of these in the original plant material has been questioned [8]. We have re-examined the heartwood, avoiding the use of ammonia and strong acids. The plant material was authenticated as described previously [7].



1a R = Me
1b R = H

RESULTS AND DISCUSSION

The powdered heartwood was subjected only to extraction with methanol and the methanol extract treated according to Brown and Warambwa [9] to achieve separation into neutral, basic and amphoteric fractions. The major alkaloid, and the only one so far obtained from the wood in sufficient quantity for identification, came from the amphoteric fraction after gel permeation chromatography, and was obtained crystalline from aqueous acetonitrile. UV data were consistent with a fully aromatic β-carboline structure and IR showed conjugated carbonyl as well as hydroxyl absorption. The 300 MHz ^{1}H NMR spectrum was of very high quality and showed only one misleading resonance: we at first interpreted a signal at *ca* δ11.9 as being caused by a carboxylic acid proton, but later work with model compounds showed that it was almost certainly attributable to the indolic N–H. The remainder of the spectrum allowed us to go some way to a structural assignment which will be explained by reference to the numbering in structure **1**. The β-carboline C-ring was represented by a singlet at δ 8.77, showing that the 5-position was substituted. There was a doublet a δ 8.36, a two-proton multiplet at *ca* 7.6 and a triplet at 7.30 showing that the benzenoid ring of the β-carboline was unsubstituted. There was clearly a substituent at C-3, and several resonances suggested that this was a seco-iridoid moiety similar to secologanin. There was a singlet at δ 7.50 corresponding to H-17 and a doublet at 5.56 for H-21. The vinyl group of secologanin-like iridoids was indicated by a doublet at δ 4.9 (J = 10 Hz), a doublet at 4.79 (J = 17 Hz), and the olefinic proton to which these were, respectively, *cis* and *trans* coupled appeared as a double triplet at 5.70.

A doublet at δ 4.59 was interpreted as the anomeric proton of a sugar, and this was supported by complex absorption in the range 2.9–3.8. There was a broad multiproton exchangeable peak at *ca* δ 5. The ^{13}C NMR spectrum showed typical resonances for a β-glucoside [10], as well as resonances for 21 other carbons, including two carbonyls, which could all be rationalized in terms of a structure similar to desoxycordifoline, **1a** [9]. On this basis all the ^{13}C NMR data could be assigned, with the proviso that some of the β-carboline assignments are uncertain. Our own data on model compounds do not support all the assignments for these carbons made by either of two earlier groups [11, 12], which are themselves in some respects contradictory. The assignments given in the Experimental are only quoted where there is definite agreement.

Methylation of the alkaloid with diazomethane gave a compound with two *O*-methyl resonances in the ^{1}H NMR spectrum, confirming the presence of two carboxylic acid groups, and implying that the original carboxyl resonances were included in the broad exchangeable peak at *ca* δ 5. The dimethyl ester of the new alkaloid proved to be identical in all respects with an authentic sample of desoxycordifoline methyl ester [9], allowing structural assignment of the new alkaloid, which we have called desoxycordifolinic acid **1b**.

After acetylation and methylation, desoxycordifolinic acid was compared with desoxycordifoline methyl ester tetra-acetate, again confirming identity. It was also poss-

ible, partially with the aid of decoupling, to assign all the peaks in the 300 MHz ^{1}H NMR spectrum of the double derivative, including the sugar protons. All the mass spectral, IR and UV data are in accord with the proposed structure (see Experimental).

It may be noted that the isolation of a 5-carboxy alkaloid of this type from a species of *Nauclea* does not support the taxonomic separation of the subtribe Adininae from the subtribe Naucleinae [13]. It is clear from the present work that such alkaloids are not restricted to Adininae. It is also possible that the adoption of suitable extraction procedures will show that the retention of glucose, a major characteristic of Adininae alkaloids [13], may be a feature of Naucleinae alkaloids as well. In this respect, the use of ammonia and/or strong acid with highly labile seco-iridoid moieties not only ensures the production of artefacts but may also obscure the true nature of the alkaloids present naturally, by removing glucose residues.

So far we have not identified any pyridine-containing alkaloids from *N. diderrichii* but we are continuing our efforts to identify the tetrahydro-β-carbolines which have been isolated in small quantities from the same extract, and which again appear to retain sugar moieties.

EXPERIMENTAL

Powdered heartwood of *N. diderrichii* (De Wild.) Merr. (*Sarcocephalus diderrichii* De Wild) (4 kg) was exhaustively percolated with cold MeOH. The extract was evaporated to give a brown powder (217 g). The powder (100 g) dissolved in MeOH (300 ml) and passed through a column of Amberlyst A15 resin, eluting first with MeOH and then with 10% Et_3N in MeOH, the latter giving a brown powder (30 g) after evaporation. This material was passed through a column of Amberlyst A26 resin, eluting first with MeOH to give a basic fraction (8.2 g) and then with 10% HOAc in MeOH to give an 'amphoteric' fraction (21 g). The 'amphoteric' fraction (10 g) was passed through a Sephadex LH20 column, eluting with MeOH. Contiguous fractions with UV and TLC indications of an alkaloidal nature were bulked to give a dark brown powder (5.9 g). This powder (0.4 g) was shaken with 80% aq. MeCN (25 ml), the clear soln decanted and left in the dark for 48 hr. Desoxycordifolinic acid was obtained as brown rosettes (56 mg), mp 206–208° (dec.). $[\alpha]_D$ −45.7° (MeOH; *c* 0.126) $\Delta\varepsilon_{242}$ +2.09, $\Delta\varepsilon_{267}$ −11.9, $\Delta\varepsilon_{290}$ −2.28, $\Delta\varepsilon_{303}$ −1.22, $\Delta\varepsilon_{376}$ −1.25 (MeOH; *c* 0.160) IR ν_{max}^{KCl} cm^{-1}: 1650 (> C = O). UV λ_{max}^{EtOH} nm: 216, 236, 272, 306sh, 354. ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.93 (1H, *s*, NH), 8.77 (1H, *s*, H-6), 8.36 (1H, *d*, *J* = 8 Hz, H-9) 7.62 (1H, *m*, H-12), 7.58 (1H, *m*, H-11), 7.50 (1H, *s*, H-17), 7.30 (1H, *t*, *J* = 7 Hz, H-10), 5. 7 (1H, *dt*, *J* = 10, 17 Hz, H-19), 5.56 (1H, *d*, *J* = 5 Hz, H-21), 4.90 (1H, *d*, *J* = 10 Hz, H-18a), 4.79 (1H, *d*, *J* = 17 Hz, H-18b), 4.59 (1H, *d*, *J* = 8 Hz, H-1), 3.69 (2H, *m*, H-6' a,b), 3.45 (1H, *m*, H-15), 3.07–3.25 (6H, *m*, H-14a,b, H-2', H-3', H-4', H-5'), 2.64 (1H, *m*, H-20). ^{13}C NMR (20.1 MHz, DMSO-d_6): δ 167.6 (*s*, COOH), 151.1 (*d*, C-17), 143.4 (*s*, C-3), 140.4 (*s*), 136.0 (*s*), 135.7 (*s*, C-5), 134.1 (*d*, C-19), 128.0 (*d*), 127.0 (*s*), 121.7 (*d*, C-9), 121.0 (*s*), 119.8 (*d*), 118.2 (*t*, C-18), 114.8 (*d*, C-6) 112.0 (*d*, C-12), 110.1 (*s*, C-16), 98.3 (*d*, C-1'), 95.4 (*d*, C-21), 77.1 (*d*, C-5'), 76.5 (*d*, C-3'), 72.9 (*d*, C-2'), 69.9 (*d*, C-4'), 60.9 (*t*, C-6'), 43.3 (*d*, C-20), 32.8 (*t*, C-14), 30.4 (*d*, C-15). EIMS, 70 eV *m/z* (rel. int.): 332 [M − glu − COOH]$^+$ (3), 314 (1), 288 (6), 270 (6), 259 (3), 243 (4), 226 (5), 205 (3), 182 (23), 179 (1), 154 (4), 126 (15), 97 (15), 44 (100). CIMS (NH_3) *m/z* (rel. int.): 333 (36), 315 (12), 289 (72), 271 (13), 227 (100), 198 (11), 183 (33), 180 (81), 162 (13), 144 (39), 127 (79). Found C, 56.0; H, 5.2; N, 4.8%. $C_{27}H_{28}N_2O_{11}\cdot H_2O$ requires C, 56.4; H, 5.2; N, 4.9%. Treatment of a MeOH soln of desoxycordifolinic acid with CH_2N_2–Et_2O followed by prep. TLC on Si gel gave di Me desoxycordifolinate mp 144–146°, mmp with Me desoxycordifoline, 145–147°, UV, IR, ^{1}H NMR and MS in accord with structure. Acetylation (Ac_2O, pyridine) followed by methylation and prep. TLC on Si gel gave diMe desoxycordifolinate tetra-acetate, mp 102–104°, mmp with methyl desoxycordifoline tetra-acetate, 101–104°. UV λ_{max}^{MeOH} nm: 235, 274, 318, 337, 350. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1750, 1680. ^{1}H NMR (300 MHz, $CDCl_3$): δ10.82 (1H, *s*, NH), 8.83 (1H, *s*, H-6), 8.22 (1H, *d*, *J* = 8 Hz, H-9), 7.72 (1H, *d*, *J* = 8 Hz, H-12), 7.63 (1H, *t*, *J* = 8 Hz, H-11), 7.54 (1H, *s*, H-17), 7.38 (1H, *t*, *J* = 8 Hz, H-10), 5.89 (1H, *dt*, *J* = 10, 17 Hz, H-19), 5.50 (1H, *d*, *J* = 5 Hz, H-21), 5.23 (1H, *d*, *J* = 17 Hz, H-18a), 5.23 (1H, *t*, *J* = 8.5 Hz, H-3'), 5.22 (1H, *d*, *J* = 10 Hz, H-18b), 5.06 (1H, *t*, *J* = 10 Hz, H-4'), 4.90–4.91 (2H, *m*, H-1', H-2'), 4.30 (1H, *dd*, *J* = 12, 4.5 Hz, H-6'a), 4.15 (1H, *dd*, *J* = 12, 2 Hz, H-6'b), 4.05 (3H, *s*, OMe), 3.90 (3H, *s*, OMe), 3.76 (1H, *m*, H-5'), 3.69 (1H, *d*, *J* = 14 Hz, H-14a), 3.35 (1H, *dd*, *J* = 14, 10 Hz, H-14b), 3.23 (1H, *dd*, *J* = 10, 5 Hz, H-15), 2.67 (1H, *dt*, *J* = 10, 5 Hz, H-20), 1.97, 1.98, 2.02, 2.10 (12H, 4*s*, 4 × COMe). EIMS, 70 eV *m/z* (rel. int.): 752.2449 [M]$^+$. Calc. for $C_{37}H_{40}N_2O_{15}$: 752.2428 (5), 421.1398; calc. for $C_{23}H_{21}O_6$: 421.1399 (19), 405.1449; calc. for $C_{23}H_{21}N_2O_5$: 405.1450 (27), 376 (5), 335 (12), 331 (6), 240 (27), 169 (57), 165 (6), 139 (5), 127 (14), 115 (12), 109 (44), 85 (58), 83 (100), 47 (18), 43 (86).

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REFERENCES

1. McLean, S. and Murray, D. G. (1970) *Can. J. Chem.* **48**, 867.
2. McLean, S. and Murray, D. G. (1972) *Can. J. Chem.* **50**, 1478.
3. Murray, D. G., Szakolcai, A. and McLean, S. (1972) *Can. J. Chem.* **50**, 1486.
4. Dmitrienko, G. I., Murray, D. G. and McLean, S. (1974) *Tetrahedron Letters* 1961.
5. Dmitrienko, G. I., Szakolcai, A. and McLean, S. (1974) *Tetrahedron Letters* 2599.
6. McLean, S. and Murray, D. G. (1972) *Can. J. Chem.* **50**, 1496.
7. Adeoye, A. O., and Waigh, R. D. (1983) *Phytochemistry* **22**, 975.
8. Hemingway, S. R. and Phillipson, J. D. (1979) in *Indole and Biogenetically Related Alkaloids* (Phillipson, J. D and Zenk, M. H., eds.) p. 70. Academic Press, New York.
9. Brown, R. T. and Warambwa, B. F. M. (1978) *Phytochemistry* **17**, 1686.
10. Bock, K., Jensen, S. R. and Nielsen, B. J. (1976) *Acta Chem. Scand. Ser. B* **30**, 743.
11. Wagner, H., Nestler, T. and Neszmelyi, A. (1978) *Tetrahedron Letters* 2777.
12. Coune, C. A., Angenot, L. J. G. and Denoel, J. (1980) *Phytochemistry* **19**, 2009.
13. Phillipson, J. D., Hemingway, S. R. and Ridsdale, C. E. (1982) *J. Nat. Prod.* **45**, 145.

Constituents of the Stems of *Arbutus unedo*

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Abstract: The dried stems of *Arbutus unedo* have been investigated for secondary metabolites. In addition to the previously reported lupeol, ursolic acid, monotropein, unedoside, and stilbericoside, the iridoids geniposide (2) and monotropein methyl ester (1) have been isolated for the first time from this source. Betulinic acid (4) has also been isolated for the first time from this plant.

Introduction

The commercial importance of *Arbutus unedo* in herbal medicine, based on a reputation for astringent, diuretic, and antiseptic properties (1) has resulted in a considerable amount of phytochemical work. This has led to the isolation of the iridoids monotropein (2), stilbericoside (3), unedide (2), and unedoside (4), all from the aerial parts. The phenolic glucoside arbutin and its methyl ether have been found in the leaves (5), as has the ubiquitous lupeol (6). Other isolated constituents include anthocyanins from the fruits (7), glycolic acid (8), and carotenoids (9), and "urson" (10) from the leaves. Sosa has reported arbutolic acid, unedosterol, ursolic acid, and a variety of other less specific products (11). Several flavonoids have recently been reported from the leaves (12).

The rationale behind our re-investigation of a plant which has already been thoroughly examined mainly concerned the iridoid content of the stems. When found, iridoids are often present as complex mixtures which cannot be separated by normal open-column chromatographic methods. We have therefore used small-scale preparative HPLC for the separation of the iridoids, but having made extracts, we also examined the material for other secondary metabolites.

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Material and Methods

The mass spectra were measured at 70 eV by direct inlet on a Kratos M-25 spectrometer fitted with a DS 55 computer data output. ^{1}H-NMR spectra were determined on a Bruker WP 80 Spectrospin (80 MHz). ^{13}C-NMR spectra were determined on the same spectrometer at a frequency of 20.1 MHz.

Isolation

The aerial parts of the plant were collected from Mount Parnis, near Athens, Greece. The material was air-dried and voucher specimens deposited with the Manchester University herbarium, where the material was authenticated.

The leaves were separated, the stems divided into bark and wood and each was powdered before being extracted successively with petrol (b. p. 40–60° C), $CHCl_3$ and MeOH in a Soxhlet apparatus.

The $CHCl_3$ extract of the bark was itself extracted with both petrol and water. The residue after this treatment was chromatographed on silica gel to give betulinic acid (4; 50 mg) m. p. 275° C (lit. m. p. 275–278° C), identified by comparison of IR and NMR spectra with those published (13) and by comparison of ^{13}C-NMR data (14).

As expected, the most interesting results were obtained from the MeOH extracts. The crude extract from the heartwood (70 g from 680 g wood) was treated according to the method of Davini (2), in which the extract is adsorbed on charcoal and extracted successively with increasing concentrations of aqueous EtOH. Extraction with 30% aqueous EtOH gave a mixture of glycosides (4 g) which was first subjected to preparative TLC on silica gel using CH_2Cl_2 – MeOH – H_2O (40 : 10 : 1) to give impure monotropein (22 mg) and an iridoid mixture (20 mg).

Preparative HPLC of both fractions on an octadecylsilane column (Spherisorb 5 ODS2), eluting with MeCN – H_2O (10 : 90), gave pure monotropein (15 mg), monotropein methyl ester (1; 12 mg) and geniposide (2; 6 mg). The structures of monotropein methyl ester and geniposide were elucidated by derivatisation and spectroscopic analysis.

Similar treatment of the MeOH extract from the bark gave a mixture of glycosides (1.3 g), which ultimately gave two further iridoids, unedoside (25 mg) and stilbericoside (27 mg).

The iridoids were identified from spectral data, as given.

Methyl ester of monotropein (1)

Colourless gum; UV: (MeOH) λ_{max} 233 nm, ^{1}H-NMR see Table I. CIMS *m/z* (rel. int. %), 422 (M + NH_4^+; 22.0), 404 (M^+; 9.1), 242 (43.9), 225 (100), 207 (77.9), 193 (69.4), 180 (91.5), 167 (64.4). ^{13}C-NMR see Table II.

Table I. ^{1}H-NMR spectral data of compounds **1, 2, 3**, (80 MHz, D_2O as solvent for 1, 2; $CDCl_3$ as solvent for 3)

	1	2	3
H-1	5.53 (d)	5.25 (d)	5.16 (d)
H-3	7.30 (br. s)	7.48 (d)	7.38 (d)
H-5	3.30 (m)	3.82 (m)	3.23 (m)
H-6	6.10 (dd)	–	–
H-7	5.61 (dd)	5.75 (m)	5.88 (m)
H-9	2.63 (dd)	–	–
H-10	3.54 (br. s)	4.20 (m)	4.72 (s-like)
O-Me	3.62 (s)	3.67 (s)	3.71 (s)
OAc	–	–	1.96–2.09 (15H)

Table II. ^{13}C-NMR chemical shift data of compound **1** (80 MHz, D_2O, dioxan as internal standard)

C–1	94.40	C– 8	84.73	C–1′	98.36
C–3	151.19	C– 9	44.06	C–2′	72.71
C–4	110.30	C–10	69.59	C–3′	76.28
C–5	37.07	C–11	169.32	C–4′	72.52
C–6	137.04	C–12	51.81	C–5′	75.70
C–7	132.09			C–6′	60.73

Geniposide (2)

Yellow gum; UV: (MeOH) λ_{max} 236.5 nm; ^{1}H-NMR see Table I. CIMS *m/z* (rel. int. %) 404 (M^+ + NH_3; 6.0), 387 (M–1; 7.9), 242 (59.7), 225 (100), 209 (22.4), 207 (83.3), 205 (89.0), 193 (25.5), 180 (79.8), 175 (45.1).

Monotropein pentacetate

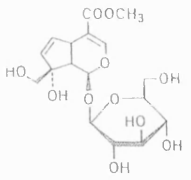
Colourless gum; ^{1}H-NMR ($CDCl_3$), 7.46 (1H, br. s, H-3), 6.26 (1H, dd, H-6), 5.63 (1H, dd, H-7), 5.57 (1H, d, H-1), 5.17 (1H, d, anomeric H), 3.76–5.10 (m sugar protons), 3.74 (2H, br. s, H-10), 3.48–3.61 (1H, m, H-5), 2.68 (1H, dd, H-9), 1.98–2.11 (12H, m). CIMS *m/z* (rel. int. %), 600 (M^+; 7.8), 583 (M^+–OH; 4.1), 366 (23.8), 331 (74.9), 213 (30.8), 186 (28.9), 169 (17.0).

Geniposide pentacetate (3)

Yellow gum; ^{1}H-NMR see Table I. CIMS *m/z* (rel. int. %), 615 (M^+ + NH_3; 3.9), 597 (M–1; 4.1), 366 (330 + $2NH_4^+$) (13.2), 331 (54.2), 289 (5.0), 271 (10.2), 249 (19.5), 229 (7.4; sugar moieties) 205 (21.2), 191 (24.6), 80 (100).

Betulinic acid (4)

^{13}C-NMR see Table III; EIMS *m/z* (rel. int. %) 456 (11.1), 438 (6.2), 423 (5.3), 410 (2.9),



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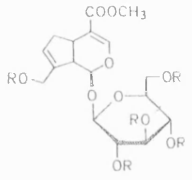

2 R = H
3 R = CO–CH_3

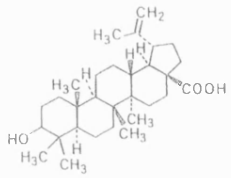

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Table III. ^{13}C-NMR chemical shift data of compound 4 (80 MHz), $CDCl_3$–C_5D_5N 1 : 1)

C– 1	38.97	C–16	32.56
C– 2	27.69	C–17	56.28
C– 3	78.65	C–18	47.19
C– 4	38.97	C–19	50.17
C– 5	55.63	C–20	150.81
C– 6	18.43	C–21	29.89
C– 7	34.53	C–22	37.23
C– 8	39.74	C–23	28.21
C– 9	50.69	C–24	15.73
C–10	37.23	C–25	16.08
C–11	20.92	C–26	16.08
C–12	25.75	C–27	14.62
C–13	38.28	C–28	179.19
C–14	43.18	C–29	19.35
C–15	30.88	C–30	109.35

395 (3.5), 302 (4.6), 259 (6.1), 248 (36.2), 207 (47.5), 189 (100.0), 175 (32.6), 135 (56.4), 119 (44.4), 107 (40.8), 95 (56.4), 81 (59.7), 69 (60.5), 55 (65.1), 43 (52.7), 43 (20.8).

Methylation of Monotropein

Monotropein (10 mg) was dissolved in MeOH (5 ml) treated with excess diazomethane in ether at 0° C and the solution taken to dryness. The residue chromatographed on silica gel with CH_2Cl_2 – MeOH – H_2O (40 : 10 : 1) afforded the pure methyl ether of monotropein (1, 4 mg), identical with the isolated material.

Acetylation of Iridoids

The iridoid glucosides (5–10 mg) were treated with dry C_5H_5N and Ac_2O for 2 h at room temperature. MeOH was added and after 15 min the solvent was evaporated. The pentacetates were isolated by preparative TLC using the system Et_2O – EtOAc (9 : 1).

Acknowledgements

We wish to thank Dr. E. Davim for a generous sample of monotropein, and Professor S. Philianos for his interest.

References

(1) Grieve, M. (1967) A Modern Herbal Vol. I. p. 53, Hafner Publishing Co., New York and London.
(2) Davini, E., Esposito, P., Iavarone, C., Trogolo, C. (1981) Phytochemistry 20, 1583–1585.
(3) Davini, E., Esposito, P., Iovarone, C., Sen, A., Trogolo, C., and Villa, S. (1981) Int. Conf. Chem. Biotechnol. Biol. Act. Nat. Prod., (Proc) 1st, 3 326–331. Edited by Atanasova, B. Bulg. Acad. Sci. Sofia Bulgaria; Ref (1982) C. A. 97, 69277r.
(4) Geissmann, T. A., Knaack, W. F., Jr., Knight, J. O. (1966) Tetrahedron Lett. 1245–1249.
(5) Ghodsi, M. R., Netien, G. (1970) Bull. Trav. Soc. Pharm. Lyon 14, 115–118.
(6) Sosa, A. (1962) Bull. Soc. Chim. Biol. 45, 117–126.
(7) Drolic, A., Raymond, J. (1981) J. Plant. Med. Phytother. 15, 109–112.
(8) Balausard, J. (1951) J. Plant. Med. Phytother. 638–639.
(9) Balausard, J. (1934) J. Plant. Med. Phytother. 1782–1785.
(10) Sanna, A. (1932) Atti IV Congr. naz. chim. pura applicata, 595–596; Ref. (1933) CA: 26 782, 4656.
(11) Sosa, A. (1950) Compt. rend. 230, 1205–1207.
(12) Dauguet, J. C., Foucher, J. P. (1982) J. Plant. Med. Phytother. 16, 185–191.
(13) Robinson, F. P., Martel, H. (1970) Phytochemistry 9, 907–909.
(14) Sholichin, M., Yamasaki, K., Kasai, R., Tanaka, D. (1980) Chem. Pharm. Bull. 28, 1006–1008.

Table 1. Effect of isopimpinellin (**1**) and 8-MOP (**2**) on viruses and cells

Organism	Fraction pfu/cfu[a] Remaining	
	Isopimpinellin (**1**)	8-MOP (**2**)
Phage T4	0.78	$< 10^{-5}$
Sindbis virus	0.90	4×10^{-3}
MCMV	0.64	$< 10^{-5}$
3T3 cells	0.82	$< 10^{-5}$

[a] Virus infectivity was assessed as plaque-forming units; pfu; cell viability as colony-forming units, cfu.
Initial values (1.00) represented 1×10^8 pfu/ml of virus and 1×10^6 cfu/ml of cells. All values were approximately 1.00 in the absence of UVA. Concentrations of compounds were 10 μg/ml. Irradiation was for 20 min.

are either quickly repaired or they do not interfere with the normal template activities of DNA or RNA. If this is the case, then the type of DNA cross-link formed by isopimpinellin must be quite different from the characteristic biadduct formed by 8-MOP. Thus, further investigations will have to focus on the type of interaction that occurs between DNA and isopimpinellin.

References

(1) Towers, G. H. N. (1984) Can. J. Bot. 62, 2900–2911.
(2) Song, P. S., Tapley, K. J. (1979) Photochem. Photobiol. 29, 1177–1197.
(3) Poulton, G. A., Ashwood-Smith, M. J. (1983) in: Carcinogens and Mutagens in the Environment, Vol. III, (Stich, H. F., ed.) CRC Press Inc., Florida 87–97.
(4) Van der Sluis, W. G., Van Arkel, J., Fischer, F. C., Labadie, R. P. (1981) J. Chromotog. 214, 349–359.
(5) Marchant, Y. Y., Turman, M., Flynn, T., Balza, F., Mitchell, J. C., Towers, G. H. N. (1985) Contact Dermatitis 12, 196–199.
(6) Abel, G., Erdelmeier, C., Meier, B., Sticher, O. (1985) Planta Med. 250–252.
(7) Altamirano-Dimas, M., Hudson, J. B., Towers, G. H. N. (1986) Photochem. Photobiol. 44, 187–192.
(8) Warren, R. A. J., Hudson, J. B., Downum, K., Graham, E. A., Norton, R., Towers, G. H. N. (1980) Photobioch. Photobiophys. 1, 385–389.
(9) Hudson, J. B., Graham, E. A., Chan, G. C., Towers, G. H. N. (1985) Photochem. Photobiol. 42, 525–528.
(10) Hudson, J. B., Graham, E. A., Towers, G. H. N. (1982) Photochem. Photobiol. 36, 181–185.
(11) Hudson, J. B., Graham, E. A., Towers, G. H. N. (1986) Photochem. Photobiol. 43, 21–26.

Isolation of Piceoside from *Arctostaphylos uva-ursi*

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The dried leaves of *Arctostaphylos uva-ursi* (Ericaceae) have been re-investigated for secondary metabolites. In addition to the previously reported arbutin and methyl arbutin the phenolic glucoside piceoside (**1**) has been isolated for the first time from this plant and from the family Ericaceae. ^{13}C-NMR data are reported for piceoside and its acetate.

The commercial importance of *Arctostaphylos uva-ursi* is based on a reputation for astringent properties and beneficial effects in nephritis and other diseases of the urinary system (1). A considerable amount of research on the plant has led to the isolation of triterpenoids (2), iridoids (3, 4), and, recently, flavonoids (5), as well as arbutin and methylarbutin (6).

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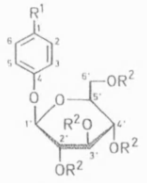

1 $R^1 = H_3C{-}CO$; $R^2 = H$
2 $R^1 = R^2 = H_3C{-}CO$

Materials and Methods

The leaves were from a commercial source, originating in Spain; a sample has been deposited with the Manchester University herbarium, where the material was authenticated.

IR spectra were recorded on a Pye-Unicam SP3-100 spectrometer in chloroform. Mass spectra were measured at 70eV by direct inlet on a Kratos MS-25 spectrometer fitted with a DS55 computer data output. ^{1}H-NMR spectra were determined on a Bruker WP80 Spectrospin (80 MHz). ^{13}C-NMR spectra were determined on the same spectrometer at a frequency of 20.1 MHz.

The leaves (350 g) were powdered before being extracted successively with petroleum ether (b.p. 40–60° C), $CHCl_3$, and MeOH in a Soxhlet apparatus. The MeOH extract was treated according to the method (7) in which the extract is absorbed on decolourising charcoal (which has previously been boiled for 15 min with distilled water) amd extracted successively with increasing concentrations of aqueous EtOH. Extraction with 30% aqueous EtOH gave a mixture of phenolic glucosides (3 g) which was first subjected to preparative TLC on silica gel using CH_2Cl_2-MeOH-H_2O (40 : 10 : 1) to give pure arbutin (40 mg), methylarbutin (20 mg), and a glucosidic mixture.

Preparative HPLC of this glucosidic mixture on an octadecylsilane column (Spherisorb 5 ODS 2) eluting with MeCN–H_2O (35 : 65) gave pure piceoside (**1**) and traces of methylarbutin. Their structures have been established by spectral data (UV, IR, ^{1}H-NMR, ^{13}C-NMR) of the glucosides and glucoside acetates. The isolated arbutin was compared with a commercial sample and the penta-acetates of both were found to be identical. Synthetic methylarbutin was found to be identical to the isolated glucoside. Piceoside tetra-acetate (**2**) was synthesised directly from 4-hydroxyacetophenone and acetobromoglucose, and found to be identical to the product of acetylation of compound **1**.

Piceoside (**1**): White crystals from MeOH; m.p. 160–165° C; UV(MeOH)λ_{max} 205, 262 nm; ^{1}H-NMR (D_2O) 7.8–7.9 (2H, d, $J = 8.2$ Hz) 7.0–7.1 (2H, d, $J = 8.2$ Hz) 5.0 (1H, d, anomeric H) 3.3–4.8 (m, sugar protons), 2.47 (3H, s); ^{13}C-NMR see Table 1; CIMS *m/z* (rel. int. %): 316 ($M + NH_4^+$; 7.6), 180 (68.1), 154 (48.5), 138 (49.0), 137 (100.0), 124 (43.6).

Table 1. ^{13}C-NMR spectral data of compounds 1 (20.1 MHz, D_2O in ppm), and 2 ($CDCl_3$)

C-Atom	1	2
C-1	NS	132.6
C-2	131.0	130.5
C-3	116.1	116.5
C-4	157.4	160.4
C-5	116.1	116.5
C-6	131.0	130.5
C-1	99.5	98.4
C-2	72.9	72.8
C-3	76.2	72.4
C-4	69.4	68.5
C-5	75.5	71.4
C-6	60.6	62.1
PhCO-	NS	196.5
$PhCOCH_3$	26.0	26.3
$O\text{-}COCH_3$	–	169.2
		169.4
		170.1
		170.5
$OCOCH_3$		20.5

NS = Not seen.

Piceoside tetracetate (2): White crystals from EtOH; m.p. 126° C; ^{1}H-NMR ($CDCl_3$) 7.87–7.98 (2H, d, J = 8.8 Hz), 6.97–7.08 (2H, d, J = 8.8 Hz), 5.2 (1H, d, J = 4.4 Hz, anomeric H), 3.77–4.27 (m, sugar protons), 2.56 (3H, s), 2.06–2.04 (12H, m); ^{13}C-NMR see Table 1; CIMS *m/z* (rel. int. %); 484 ($M + NH_4^+$; 12.4), 33.1 (68.0), 137 (100.0).

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References

(1) Lewis, W. H., Elvin-Lewis, M. P. F. (1977) Medical Botany, J. Wiley and Sons, p. 361.
(2) Malterud, K. E. (1980) Medd. Nor. Farm. Selsk. 42, 15–20; Ref.(1980) C. A. 93, 146372.
(3) Briggs, L. H., Cain, B. F., Le Quesne, P. W., Shoolery, J. N. (1963) Tetrahedron Lett. 69–74.
(4) Jahodar, L., Leifertova, I., Lisa, M. (1978) Pharmazie 33, 536–538.
(5) Geiger, H., Schuecker, U., Waldrum, H., Vahder Velde, G., Mabry, T. Jr. (1975) Z. Naturforsch. 30c, 296; Ref (1975) C. A. 83, 4956.
(6) Ahtardjieff, Chr. (1966) Pharmazie 21, 59–60.
(7) Davini, E., Esposito, P., Iavarone, C., Trogolo, C. (1981) Phytochemistry 20, 1583–1585.
(8) Entlicher, G., Kocourek, J. (1967) Arch. Biochem. Biophys. 118, 305–309.

Major Cardenolide Glycosides in Embryogenic Suspension Cultures of Digitalis lanata

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It is well known that undifferentiated cell cultures of *Digitalis lanata* Erh. (Scrophulariaceae) are not able to produce cardenolides (1, 2), whereas morphological differentiation leads to cardiac glycoside formation (3, 4, 5). The relative cardenolide content is usually measured as digitoxin or digoxin equivalents by radioimmunological methods (4, 5).

We now wish to report the isolation and identification of five major cardiac glycosides from an embryogenic cell strain of *Digitalis lanata*.

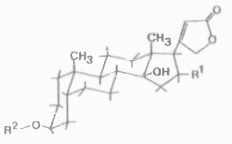
The differentiation process was initiated by transferring cells of an embryogenic long-term suspension cultured strain derived from filament to an appropriate differentiation medium. After 6 weeks cardenolide formation started (as monitored by radioimmunoassay) and reached a constant maximum of 110 µg/g dry weight after approximately 15 weeks.

At that time odorosid H, odorobiosid G, glucodigifucosid, verodoxin and strospesid (for structural formula see Table 1) could be isolated from a highly purified extract. The freeze-dried plant material was homogenized and sonically extracted with MeOH-H_2O (8 : 2) at 40° C. The filtrate was diluted with water and purified by precipitation with lead acetate (6). The cardenolides were extracted with $CHCl_3$ and $CHCl_3$ – isopropanol (3 : 2). The pooled organic phases were brought to dryness and further purified by column chromatography on RP- 18 cartridges (Baker™). The cardenolides were eluted with EtOH- H_2O (7 : 3), the solvent evaporated to dryness and the residue dissolved in MeOH. This extract was fractionated by preparative HPLC on Nucleosil RP-18 columns, 5 µm, employing a gradient from 20 to 55 % acetonitrile/water during 60 minutes (modified after (7)). Each fraction was monitored by TLC on silicagel plates developed in $CHCl_3$-MeOH-H_2O (80 : 18 : 2). In five fractions cardenolides could be positively detected by spraying with Jensen's and Keede's reagent (8). No positive colour reaction was obtained with xanthydrol, indicating that none of the cardiac glycosides contained 2,6-dideoxy sugars such as digitoxose (8). Mild acidic hydrolysis of all fractions with MeOH-1N HCl (1 : 1) at 50° C yielded no aglyca either. After direct hydrolysis on the TLC plate with HCl vapors and detection with thymol/sulfuric acid (8) digitalose, fucose and glucose were found as sugar components. The identification of the five cardenolides was verified by comparison

Tabelle 1. Main Cardiac Glycosides in Embryogenic Cultures of *Digitalis lanata*.

R^1	$R^{2(a)}$	
-H	Dtl-	Odorosid H
-H	Gluc-Dtl-	Odorobiosid G
-H	Gluc-Fuc-	Glucodigifucosid
-OCHO	Dtl-	Verodoxin
-OH	Dtl-	Strospesid

(a) Digitalose (Dtl), Fucose (Fuc), Glucose (Gluc)



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THREE DITERPENES WITH A SECOLABDANE SKELETON FROM *CLUTIA ABYSSINICA*

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Key Word Index—*Clutia abyssinica*; Euphorbiaceae; diterpenes; clutiolide; dihydroclutiolide; isodihydroclutiolide.

Abstract—The roots of *Clutia abyssinica* have yielded three new diterpenes with a 6,7-secolabdane skeleton. Detailed structural assignments have been made from high-field NMR data, particularly nuclear Overhauser effects.

INTRODUCTION

The perennial shrub *Clutia abyssinica* Jaub and Spach var. *abyssinica* (syn. *C. glabrescens* Knauf) is widespread in the drier regions of Africa. Extracts of the aerial parts are popularly used in Ethiopia to treat skin ailments [1]. Apart from our previous work on *C. abyssinica* [2, 3], there appear to be only two reports on the chemistry of the genus, secolabdane diterpenes having been reported [4, 5] from *Cluytia richardiana*. The preferred spelling is *Clutia* [6], based on the latinized form *Clutius* of the name of the Dutch botanist Cluyt.

RESULTS AND DISCUSSION

The three new diterpenes were obtained in a high state of purity from the chloroform extract of the powdered roots by means of chromatography on silica gel and repeated crystallization from methanol. As well as the usual MS and IR measurements, the compounds were subjected to NMR spectroscopy at frequencies up to 500 MHz and it is largely on these data that the structural proposals are based. The molecular formula of clutiolide (**1a**) from accurate mass measurement is $C_{20}H_{22}O_5$. In the high field ^{1}H NMR spectrum the four-spin system at positions 1-2-3 can be readily assigned because it is characterized by chemical shifts and spin–spin coupling in 1-D and COSY spectra. Similarly, the four-spin ^{1}H system at positions 9-11-12 can be assigned from the same spectra; chemical shifts and coupling are completely compatible and in agreement with data for similar compounds. The coupling constants for H-11α and H-11β with H-12 agree closely with those reported for fibraurin [7], while the chemical shifts for these protons correlate with those for the analogous system in seconidorella lactone [8], one of the few examples of a similar four-spin system. The precise figures for chemical shifts and coupling constants are given in Fig. 1, as are COSY and HETCOR (^{1}H–^{13}C) correlations. The chemical shifts and coupling constants for the protons at positions 14, 15 and 16 are characteristic for 3-substituted furans, as for example in saudin [4].

The two protons attached to position 19 resonate at δ4.02 and 4.07 in accord with their position adjacent to oxygen. The protons of the exocyclic methylene group attached to position 7 have a large chemical shift difference, δ5.51 and 6.12, consistent with their position next to the carbonyl and comparable with similar exocyclic methylenes [9]. The single proton attached to saturated carbon at δ2.26 assigned to position 5 and the two methyls at δ1.09 and 1.24 complete the assignments for all 22 protons. The higher field methyl has to be C-20 to satisfy the NOE data below.

The 2-D HETCOR plots for clutiolide were very clean and unambiguous. The correlations given in Fig. 1 show ^{13}C resonance frequencies which are completely compatible with positions in the proposed structure. The numbers of attached protons for all carbons were obtained using DEPT and are as the structure requires. The only ambiguities in the ^{13}C assignments concern the

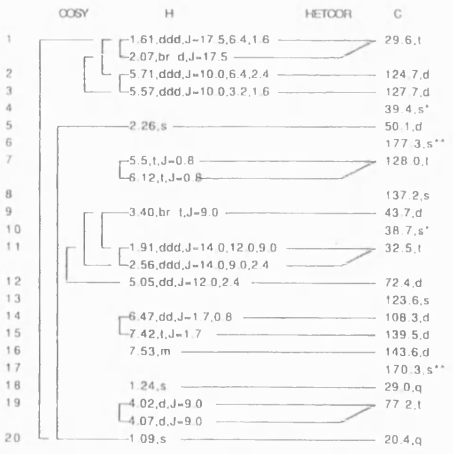


Fig. 1. NMR data for clutiolide obtained at 500 MHz. Assignments marked * and ** may be reversed.

quaternary carbons 6, 17 and 4, 10. Interchange of these assignments does not affect the structure proposal.

There are many structures which would accommodate the 1D, COSY and HETCOR data. However, the molecular formula indicates that there are 10 ring or double bond equivalents. After the double bonds are accounted for, four rings remain. One of these is the furan and another can be allocated to the γ-lactone. IR absorption at 1775 cm^{-1} tended to support this suggestion. Most of the remainder of the molecule therefore consists of two rings. Both NOESY and 1D NOE difference spectroscopy demonstrate that these are two six-membered rings as shown and allow the relative stereochemistry to be assigned.

For the sake of simplicity we have assumed that the absolute stereochemistry at position 12 is *S*, because the majority of diterpenes with similar furanolactone moieties have this configuration, as do the specifically related compounds [5] richardianidin-1 and -2. With this assumption, the configuration at position 9 is *R*, because there was a NOE between protons at positions 9 and 12 which are therefore *cis* and pseudoaxial. The relative stereochemistry within the bicyclic residue attached to position 9 is readily established. A NOE between the proton at position 5 and methyl 18 showed that the ring junction is *cis*. A further NOE between this methyl and the alkene proton at position 3 confirms the position of the double bond, as does a NOE between this alkene proton and the proton at position 19 resonating at δ4.07. A crucial NOE between this proton at position 19 and methyl 20 demonstrates that methyl 20 is *trans* to methyl 18 and to the proton at position 5. These NOE's are indicated on the stereostructure (Fig. 2).

It is more difficult to assign the stereochemistry of the bicyclic residue relative to that of the δ-lactone to which it is attached. However, assuming that the bicycle prefers to be pseudoequatorial relative to the δ-lactone, and that the δ-lactone prefers to be pseudo-equatorial relative to the cyclohexane ring, there are several interactions between protons on both residues which help to establish the relative configuration. The ring junction proton at position 5 has a pivotal role in view of the numerous NOE's in which it is involved (Fig. 2). In order to satisfy these and those involving protons at position 1 with position 11 and methyl 20 with position 9, a conformation is required similar to that shown in Fig. 2, in which the dihedral angle 1-10-9-11 is approximately 60°. If the relative configuration between positions 9 and 10 is reversed, NOEs as observed result in severe steric interactions between the exocyclic methylene group (position 7) and the carbonyl at position 6.

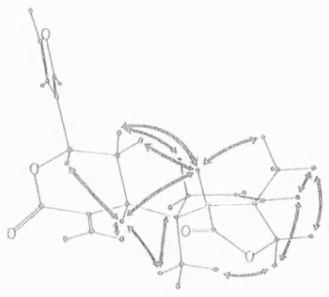


Fig. 2. Significant NOE's observed for clutiolide at 500 MHz; a combination of 1D and 2D data.

The analogue **1b** has a molecular formula $C_{20}H_{24}O_5$, a gain of two hydrogens. These are readily assigned to the 2 and 3 positions from NMR data, the alkene protons at δ5.57 and 5.71 having disappeared and the alkane region having gained. The other ^1H chemical shifts are similar to those for **1a**. The ^{13}C data including multiplicities are all consistent with the proposed structure and are given along with COSY connections and ^1H data in Fig. 3.

The NOE data for **1b** confirmed the relative stereochemistry to be similar to that of **1a**. Of particular significance are NOEs between Me-20 and the β proton attached to position 19, between Me-18 and the α proton attached to position 19 and between Me-18 and the ring junction proton at position 5. A NOE between Me-20 and the single proton at position 9 offers further support for the stereochemistry assigned to both **1a** and **1b**. Attempts to convert **1a** to **1b** by hydrogenation have so far given complex mixtures.

The isomer **2** of the dihydro compound **1b** showed two features in the ^1H NMR spectrum which are of immediate significance. The pattern for the two protons at *ca* δ4, previously an AB quartet in both **1a** and **1b**, became a pair of triplets showing that both protons of the quartet are coupled to a third proton. This third proton resonated at δ2.48 and corresponds to the proton, previously a singlet in **1a** and **1b**, resonating at δ2.26 and 2.08 respectively. On this basis it is not difficult to propose structure **2** for the isomer of **1b** and this assignment is supported by all the ^1H data, including coupling constants and chemical shifts, by COSY, ^{13}C chemical shifts and multiplicities and by HETCOR correlations (Fig. 4). The NOE data are particularly helpful, because they confirm some of the spatial relationships proposed for **1a** and **1b**, as follows.

A NOE between protons at positions 9 and 12 in isomer **2** again confirms the *cis* orientation of the two

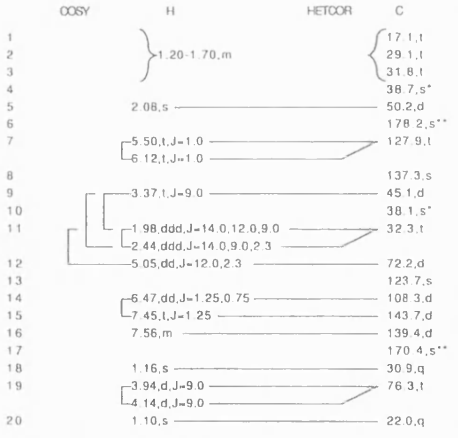


Fig. 3. NMR data for dihydroclutiolide obtained at 300 MHz. Assignments marked * and ** may be reversed.

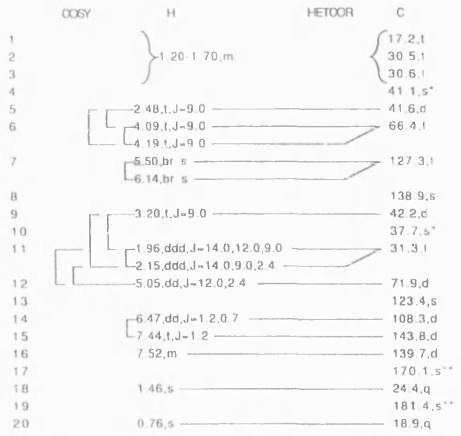
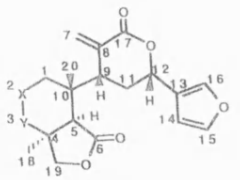
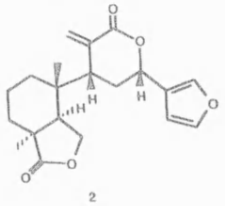


Fig. 4. NMR data for isodihydroclutiolide obtained at 300 MHz. Assignments marked * and ** may be reversed.

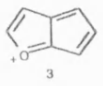
bulky substituents on the δ-lactone. From a NOE between the protons at positions 5 and 9 it can be deduced that the δ-lactone and the proton at position 5 are *cis* with respect to the cyclohexane ring. This proton and methyl 18 are also *cis*, from the presence of a NOESY cross-peak. Methyl 18 and the δ-lactone must therefore be *cis*, and this is confirmed by the existence of NOESY cross-peaks between methyl 18 and the higher field proton at position 7, the exocyclic methylene. In isomer **2** a conformation is permitted in which the exocyclic methylene approaches position 6, there being no carbonyl at this position. When this occurs methyl 20 comes under the anisotropic shielding influence of the π-electrons of the exocyclic methylene, accounting for the marked upfield shift of this methyl, from δ1.10 in **1b** to δ0.76 in **2**.



1 a XY= -CH=CH-
b XY= $-CH_2CH_2-$



2



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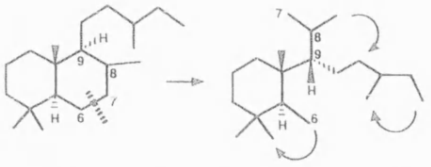

Fig. 5. Derivation of clutiolide and related diterpenes from labdane.

The electron impact mass spectra for all three compounds were consistent with the proposed structures. In particular, there is a tendency for cleavage of the 9,10 bond to give fragments, in the case of clutiolide, $C_{10}H_{10}O_3$ and $C_{10}H_{13}O_2$. For **1b** and **2** the fragment corresponding to the latter is $C_{10}H_{15}O_2$. The base peak for clutiolide, C_7H_5O, is probably derived from the furan ring and three carbons of the δ-lactone in the relatively stable bicyclic ion 3.

Our assumption that there is a biogenetic relationship between these diterpenes and labdane (Fig. 5) is consistent with the observed relative stereochemistry. Labdane is found in both mirror image forms, but if we assume 5*S*,9*S*,10*R* stereochemistry as a starting point we arrive at the stereochemistry of the present diterpenes as suggested above. This is particularly gratifying for positions 9 and 10, where free rotation can occur in the *seco*-labdanes.

EXPERIMENTAL

Mps: uncorr. ^1H (300, 400, 500 MHz) and ^{13}C (75.4 MHz) NMR: $CDCl_3$ with TMS as int. standard. MS: 70 eV. CC: silica gel 0.125–0.25 μ (70%) and silica gel for TLC type H 10–40 μ (30%). Plant material was collected in Jan. 1983 near Kibremengist, Sidamo region, Ethiopia and identified at the Herbarium of the Biology Department, Addis Ababa University, where a voucher specimen was deposited.

Extraction and isolation. Ground root of *C. abyssinica* (2 kg) was successively extracted with petrol, $CHCl_3$ and MeOH at room temp. The $CHCl_3$ extract was evapd *in vacuo* to give a brownish gummy residue (33 g) which was exhaustively washed with petrol. The petrol-insoluble material (26 g) was washed with H_2O and dried to give a brownish-yellow gum. Of this, 6 g was chromatographed on a silica gel column, eluting with petrol containing increasing amounts of EtOAc. EtOAc–petrol (1:4) gave fractions B-1, B-2 and B-3. B-1 furnished a crystalline compound (**1a**) from MeOH. B-2 and B-3 on further purification by centrifugal chromatography and repeated crystallization from MeOH yielded, crystalline **1b** and **2** respectively.

Clutiolide (**1a**) (34 mg). Mp 165–166° (MeOH); $[\alpha]_D$ −68.9° ($CHCl_3$, *c* 0.189). λ_{max}^{MeOH} nm (log ε): 210 (3.76). ν_{max}^{KBr} cm^{-1}: 1775 (γ-lactone), 1750 (δ-lactone), 1638, 1618 (C=C), 1501, 1422, 1363, 1159, 1068, 1032, 877. EIMS *m/z* (rel. int.): 342.1468 ($C_{20}H_{22}O_5$, calcd 342.1467) $[M]^+$ (4.4), 314 (5.4), 298 (5.4), 218 (6.8), 178 (48), 177 (18), 165 (35), 134 (20), 133 (55), 132 (31), 119 (56), 107 (46), 105 (100), 94 (80), 93 (88), 91 (88), 86 (61); NOEs not given in the text

(^1H–^1H): 14-15, 2-3, 1α-1β, 1β-2, 19α-19β, 11α-11β and 7a-7b.

Dihydroclutiolide (**1b**) (74 mg). Mp 119–120° (MeOH); $[\alpha]_D$ −90° ($CHCl_3$; *c* 0.186). λ_{max}^{MeOH} nm (log ε): 210 (3.78). ν_{max}^{KBr} cm^{-1}: 3147, 2935, 2876, 1763 (γ-lactone), 1750 (δ-lactone), 1638, 1618, 1502, 1458, 1386, 1364, 1238, 1056, 1010, 877. EIMS *m/z* (rel. int.): 344.1623 ($C_{20}H_{24}O_5$, calcd 344.1623) $[M]^+$ (8.9), 268 (21), 251 (8.6), 236 (7), 178 (100), 177 (22), 167 (64), 133 (70), 123 (78), 105 (46), 81 (13); NOEs not included in the text (^1H–^1H): 14-15, 9-7a, 7a-7b, 11α-11β, 19α-19β and 9-11β.

Isodihydroclutiolide (**2**) (112 mg). Mp 156–157° (MeOH); $[\alpha]_D$ −44.4° ($CHCl_3$; *c* 0.18). λ_{max}^{MeOH} nm (log ε): 211 (3.86). ν_{max}^{KBr} cm^{-1}: 3135, 2947, 2863, 1767 (γ-lactone), 1733 (δ-lactone), 1638, 1512, 1490, 1357, 1109, 1026, 876. EIMS: 344.1648 ($C_{20}H_{24}O_5$ calcd 344.1624) $[M]^+$ (0.8), 179 (13), 178 (100), 167 (46), 166 (10), 133 (41), 123 (17), 109 (65), 105 (25), 93 (36), 81 (33); NOEs not included in the text: (^1H–^1H) 11α-11β, 9-11β, 11β-12, 7a-7b and 14-15.

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REFERENCES

1. Gebre-Egziabiher, T. (1980) *Glossary of the Ethiopian Medicinal Plants*, p. 24. Addis Ababa University Press, Addis Ababa.
2. Zerihun, B., Lockwood, G. B. and Waigh, R. D. (1987) *J. Nat. Prod.* **50**, 322.
3. Zerihun, B., Lockwood, G. B. and Waigh, R. D. (1987) *Fitoterapia* **58**, 192.
4. Mossa, J. S., Cassady, J. M., Antoun, M. D., Byrn, S. R., McKenzie, A. T., Kozlowski, J. F. and Main, P. (1985) *J. Org. Chem.* **50**, 916.
5. Mossa, J. S.,Cassady, J. M., Kozlowski, J. F., Zennie, T. M., Antoun, M. D., Pellechia, M. G., McKenzie, A. T. and Byrn, S. R. (1988) *Tetrahedron Letters* **29**, 3627.
6. Palmer, E. and Pitman, N. (1972) *Trees of Southern Africa* (Vol. II), pp. 1088–1182. Balkema, Cape Town.
7. Hori, T., Kiang, A. K., Nakanishi, K., Sasaki, S. and Woods, M. C. (1967) *Tetrahedron* **23**, 2649.
8. Bohlmann, F., Wegner, P. and Jakupovic, J. (1986) *Phytochemistry* **21**, 1981.
9. You-you, T., Mu-yun, N., Yu-rong, Z., Lau-na, L., Shu-lian, L., Mu-qun, Z., Xiu-zhen, W., Zheng, J. and Xiao-tian, L. (1982) *Planta Med.* **44**, 143.

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TEN 5-METHYLCOUMARINS FROM *CLUTIA ABYSSINICA*

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Key Word Index—*Clutia abyssinica*; Euphorbiaceae; coumarins; NMR.

Abstract—The chloroform extract of the powdered whole root of *Clutia abyssinica* has yielded a complex mixture of 5-methylcoumarins, of which 10 have been isolated in pure form and identified, mostly by NMR.

INTRODUCTION

The East African shrub *Clutia abyssinica* has proved to be a rich source of secondary metabolites [1]. As a continuation of our work on the species we have made a careful examination of the chloroform extract of the powdered whole roots. This has yielded a total of 10 5-methylcoumarins and there are traces of yet more. Of the 10 which we have isolated in a pure form, seven have not been previously reported, including three which contain sulphur.

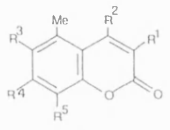
RESULTS AND DISCUSSION

Taking the non-sulphur containing compounds first, in order of increasing substitution, the simplest structure isolated was 4-methoxy-5-methylcoumarin (**1a**), also known as ekersenin [2] or pereflorin [3]; the compound was first reported with the wrong structure and without a trivial name [4]. It appears that the name ekersenin takes historical precedence. The structure was first confirmed by a simple synthesis from *meta*-cresol and malonic acid which gives a mixture of isomers [5]. The unwanted 7-methyl isomer predominates and was reportedly removed by crystallization [5]. In our hands it was easier to methylate the mixture and separate by semi-preparative HPLC.

The structure of the previously unknown 3,5-dimethyl-4-methoxycoumarin **1b** was confirmed by an analogous synthesis using methylmalonic acid. As expected, the required isomer was the minor component of the mixture of products and was again separated by semi-prep. HPLC.

Isolation of the known [6] 8-hydroxy-4-methoxy-5-methylcoumarin **1c** (8-hydroxypereflorin) was confirmed by comparison with an authentic sample. The previously unknown 3-methyl analogue **1d** was distinguished from other possible hydroxy isomers by the observation of *ortho* coupling between the aromatic protons and an NOE between the 5-methyl group and one of the aromatic protons.

Two di-oxygenated analogues presented greater difficulty in identification, because the measurement of NOEs allowed alternative possibilities. Both 3,5-dimethyl-4,6,7-trimethoxycoumarin (**1e**) and 3,5-dimethyl-4,6-dimethoxy-7-hydroxycoumarin (**1f**) showed NOEs (Figs 1 and 2), which leave open the question of whether there is oxygenation in the 7 or 8 position. This ambiguity was resolved by the measurement of ^{13}C chemical shifts for the methoxy groups, which show two methoxys out-of-plane (chemical shifts *ca* 60 ppm) for both analogues, consistent with substituents on both sides of each methoxy [7]. The 7-methoxy group in **1e** is in-plane, with a chemical shift of 56.0 ppm. Final confirmation of the structures and complete assignments for the ^{13}C spectra were obtained by a combination of 2D heteronuclear correlation experiments and long-range correlations achieved using the FLOCK pulse sequence [8]. The ^{13}C assignments for **1f** were completed by treatment of the chloroform-*d* solution with a H_2O–D_2O mixture, which caused slow exchange of the hydroxyl proton and a resultant broadening and reduction in intensity of the line for C-7. The chemical shifts and FLOCK correlations are given in Figs 1 and 2.



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	R^1	R^2	R^3	R^4	R^5
a	H	OMe	H	H	H
b	Me	OMe	H	H	H
c	H	OMe	H	H	OH
d	Me	OMe	H	H	OH
e	Me	OMe	OMe	OMe	H
f	Me	OMe	OMe	OH	H
g	H	SMe	H	H	H
h	Me	SMe	H	H	OH
i	H	SMe	OMe	OH	H
j	H	SMe	OMe	OMe	H

Four 4-methylthiocoumarins were isolated, the simplest being the known [9] 5-methyl-4-methylthiocoumarin **1g**. To confirm the structure, 4-hydroxy-5-methylcoumarin, obtained in a mixture in the first stage of the synthesis of the 4-methoxy analogues as described above, was separated by prep. TLC, tosylated and substituted with methanethiolate. This is a slight

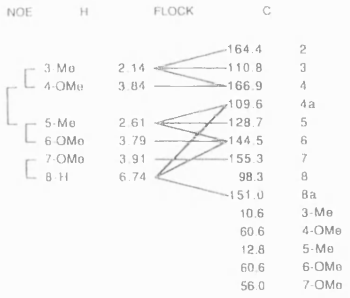

Fig. 1. NOE data and long-range (FLOCK) H–C connections for coumarin **1e**.

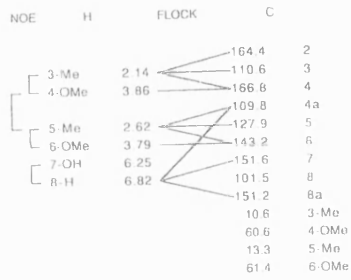

Fig. 2. NOE data and long-range (FLOCK) H–C connections for coumarin **1f**.

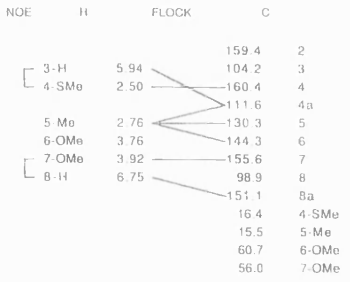

Fig. 3. NOE data and long-range (FLOCK) H–C connections for coumarin **1j**.

improvement on the later stages of the previously reported method [9].

The structure of 3,5-dimethyl-8-hydroxy-4-methylthiocoumarin **1h** was largely established from ^{1}H NMR data. Observation of a NOE between the 5-methyl group and an aromatic proton established that the hydroxy group was not at position 6. Unfortunately the aromatic protons were magnetically equivalent, preventing the measurement of coupling constants: the structure was confirmed by the observation of *ortho* coupling between the aromatic protons in the methyl ether, prepared by reaction with diazomethane.

The final two coumarins were 7-hydroxy-6-methoxy-5-methyl-4-methylthiocoumarin (**1i**) and its 7-methoxy analogue **1j**. The latter showed NOEs as in Fig. 3, and confirmation of the substitution pattern was obtained as for **1e** and **1f** by a combination of ^{13}C NMR chemical shifts, which show the 6-methoxy group to be out-of-plane, and by heteronuclear correlation, including FLOCK experiments (Fig. 3). The structure of the 7-hydroxy analogue **1i** follows from comparison of NMR data with those for **1j**, most significantly the chemical shift for the methoxy group at 61.4 ppm which is out-of-plane and therefore at position 6, while the hydroxy group has to be at position 7.

EXPERIMENTAL

Collection and preparation of plant material. Roots of *C. abyssinica* Jaub and Spach were collected in January 1983 near Kibremengist, Sidamo region, Ethiopia and identified at the Herbarium of the Biology Department, Addis Ababa University, where a voucher specimen is deposited.

Extractionation and fractionation. Ground whole root of *C. abyssinica* (2 kg) was successively extracted with petrol (bp 60–80°), $CHCl_3$ and MeOH at room temp. for 2, 3 and 4 days respectively. The $CHCl_3$ extract was evapd *in vacuo* to give a brown gummy residue (33 g) which was exhaustively washed with petrol. The petrol-insoluble material (26 g) was washed with H_2O and dried to give a brownish-yellow gum, of which 6 g was chromatographed on a silica gel column and eluted with petrol, and petrol containing increasing quantities of EtOAc. Appropriate combinations of centrifugal chromatography, semi-prep. HPLC, prep. TLC and crystallization from MeOH gave the 10 coumarins. Mps: uncorr. ^{1}H NMR spectra were recorded at 270 and 300 MHz with TMS as int. standard in $CDCl_3$. ^{13}C NMR spectra were recorded at 67.5 MHz with TMS as int. standard in $CDCl_3$. Carbon atom type ('multiplicity') was determined from broad-band decoupled and DEPT 135 experiments. HPLC separations were carried out on a Dynamax-60A normal phase column (25 cm × 10 mm i.d.) with a Waters detector at 254 nm.

4-*Methoxy*-5-*methylcoumarin* (*ekersenin, pereflorin* **1a**). Mp 152–153° (MeOH) (lit. [2] 165° [3] 115°); λ^{MeOH} nm: 274, 284, 304; ν^{KBr} cm^{-1}: 1704, 1616, 1600, 1562, 1257; EIMS: *m/z* (rel. int.): 190 (100), 175 (6.7), 162 (43), 147 (55), 132 (24); ^{1}H NMR ($CDCl_3$): 7.49–7.02 (3H, *m*, H-6, 7 and 8), 5.65 (1H, *s*, H-3), 3.94 (3H, *s*, OMe-4), 2.64 (3H, *s*, Me-5). A sample for comparison was synthesized from *m*-cresol (11.25 g) and malonic acid (12.35 g) which were heated at 60–65° with $POCl_3$ (33 ml) and $ZnCl_2$ (49 g) for 36 hr. Work-up according to the lit. method [5] gave a yellow-orange solid (9.0 g) which was a mixture of 4-hydroxy-7-methylcoumarin and the desired 4-hydroxy-5-methylcoumarin. These could be separated with difficulty, but for the 4-methoxy compound it was easier to methylate a sample with K_2CO_3–Me_2SO_4 and separate on normal phase HPLC with EtOAc–hexane (3:7), giving pure material, identical with the natural coumarin.

4-*Methoxy*-3,5-*dimethylcoumarin* (**1b**). Mp 102–103°; λ^{MeOH} nm (log ε): 217 (4.4), 283 (4.4), 314 (*sh.*, 4.15); EIMS: *m/z* (rel. int.): 204 (89), 189 (73), 161 (100), 145 (13), 135 (13), 115 (17), 105 (18), 91 (14), 77 (16); ^{1}H NMR ($CDCl_3$): 7.35–7.07 (3H, *m*, H-6, H-7 and H-8), 3.88 (3H, *s*, OMe-4), 2.70 (3H, *s*, Me-5), 2.18 (3H, *s*, Me-3). Synthetic material was obtained essentially as for **1a**,

except that methylmalonic acid was used in place of malonic acid. From *m*-cresol (4.5 g) and methylmalonic acid (5 g) the yield of mixed coumarins was 0.67 g. A sample of this mixture was methylated and separated on normal phase HPLC with EtOAc–hexane (1:4) to give **1b**, identical with the natural coumarin.

8-*Hydroxy*-4-*methoxy*-5-*methylcoumarin* (8-*hydroxypereflorin*, **1c**). Mp 234–236° (lit. [6] 230–232°); λ^{MeOH} nm: 205, 253, 285; EIMS: *m/z* (rel. int.): 206 (23.5), 191 (11.8), 179 (17.6), 178 (11.8), 163 (23.5), 149 (182.4), 105 (76.5), 97 (100), 77 (88.2); ^{1}H NMR ($CDCl_3$): 7.01 (1H, *d*, *J* = 8 Hz, H-7), 6.96 (1H, *d*, *J* = 8 Hz, H-6), 3.97 (3H, *s*, OMe-4), 2.57 (3H, *s*, Me-5).

8-*Hydroxy*-4-*methoxy*-3,5-*dimethylcoumarin* (**1d**). Mp 200–201°; λ^{MeOH} nm (log ε): 204 (3.57), 257 (3.07), 294 (3.19); EIMS: *m/z* (rel. int.): 220 (100), 205 (38), 192 (14), 177 (47), 161 (32), 149 (28), 133 (21), 105 (24), 90 (23), 77 (31); ^{1}H NMR ($CDCl_3$): 6.98 (1H, *d*, *J* = 8 Hz, H-7), 6.96 (1H, *d*, *J* = 8 Hz, H-6), 5.7 (1H, *br s*, exch., OH-8), 3.88 (3H, *s*, OMe-4), 2.61 (3H, *s*, Me-5), 2.18 (3H, *s*, Me-5).

4,6,7-*Trimethoxy*-3,5-*dimethylcoumarin* (**1e**). Mp 123–124°; λ^{MeOH} nm (log ε): 275 (3.8), 284 (3.9), 305 (3.7), 318 (3.5); ν^{KBr} cm^{-1}: 1719, 1609, 1562, 1401, 1362, 1259, 1197, 811, 741; EIMS *m/z* (rel. int.) 264 (100), 249 (79), 236 (32), 221 (69), 206 (8), 194 (10), 177 (34), 163 (18), 162 (32), 147 (23), 91 (48), 77 (38); ^{1}H and ^{13}C NMR data are given in Fig. 1; delays in the FLOCK pulse sequence [8] were Δ^1 = 46.5 msec, Δ^2 = 25.5 msec with a relaxation delay of 3.0 sec.

7-*Hydroxy*-4,6-*dimethoxy*-3,5-*dimethylcoumarin* (**1f**). Mp 199–200°; λ^{MeOH} nm (log ε): 228 (4.1), 255 (3.2), 298 (3.9), 325 (4.11); ν^{KBr} cm^{-1}: 1686, 1617, 1570, 1431, 1360, 1289, 1138, 1101, 897, 782, 743; EIMS *m/z* (rel. int.): 250 (72), 235 (45), 222 (100), 207 (69), 179 (14), 163 (10), 147 (16), 91 (21), 77 (18); ^{1}H and ^{13}C NMR data are given in Fig. 2; delays in the FLOCK pulse sequence [8] were Δ^1 = 86.5 msec, Δ^2 = 46.5 msec with a relaxation delay of 2.0 sec.

5-*Methyl*-4-*methylthiocoumarin* (**1g**). Mp 184–185° (MeOH) (lit. [9] 192.5°); λ^{MeOH} nm: 236, 271, 333 (sh); ν^{KBr} cm^{-1}: 1706, 1597, 1553, 1401, 1332, 1278, 1160, 899, 788; EIMS *m/z* (rel. int.): 206 (100), 191 (76), 178 (64), 163 (42), 159 (21), 147 (10), 134 (17), 91 (21), 77 (23); ^{1}H NMR ($CDCl_3$): 7.39–7.01 (3H, *m*, H-6, 7 and 8), 6.06 (1H, *s*, H-3), 2.86 (3H, *s*, Me-5), 2.52 (3H, *s*, Me-4). A sample was obtained for comparison by synthesis from the mixture of 4-hydroxycoumarins obtained as described under **1a**. The mixture (88 mg) in dry pyridine (3 ml) was treated with *p*-toluenesulphonylchloride (95 mg) in dry pyridine (3 ml). After 12 hr the mixture was poured onto ice and extracted with Et_2O. The tosylate (50 mg) was treated with sodium thiomethoxide (15 mg) in dry pyridine (3 ml) for 24 hr, the pyridine removed under vacuum and the products separated by prep. TLC on silica gel using Et_2O–petrol (2:3) to give **1g** (8 mg), identical with the natural product.

8-*Hydroxy*-3,5-*dimethyl*-4-*methylthiocoumarin* (**1h**). Mp 174–175°; λ^{MeOH} nm (log ε): 265 (4.02), 309 (4.01); ν^{KBr} cm^{-1}: 1674, 1611, 1548, 1489, 1401, 1356, 1248, 1139, 837, 764; EIMS *m/z* (rel. int.): 236 (100), 221 (17), 208 (24), 193 (55), 162 (49), 147 (55), 132 (32), 103 (29), 91 (41), 77 (55); ^{1}H NMR ($CDCl_3$): 6.98 (2H, *s*, H-6 and H-7), 2.81 (3H, *s*, Me-5), 2.47 (3H, *s*, Me-3), 2.34 (3H, *s*, SMe-4). Treatment with diazomethane gave 3,5-dimethyl-8-methoxy-4-methylthiocoumarin as an oil: EIMS *m/z* (rel. int.): 250 (100), 222 (31.0), 175 (27.6), 138 (69.0), 121 (72.4), 103 (69.0), 77 (79.3); ^{1}H NMR ($CDCl_3$): 6.97 (1H, *d*, *J* = 8 Hz, H-7), 6.94 (1H, *d*, *J* = 8 Hz, H-6), 3.92 (3H, *s*, 8-OMe), 2.81 (3H, *s*, Me-5), 2.47 (3H, *s*, Me-3), 2.34 (3H, *s*, SMe-4).

7-*Hydroxy*-6-*methoxy*-5-*methyl*-4-*methylthiocoumarin* (**1i**). Mp 224–225°; λ^{MeOH} nm (log ε): 221 (4.02), 284 (3.83), 308 (3.85), 331 (3.86); ν^{KBr} cm^{-1}: 1703, 1613, 1526, 1401, 1365, 1275, 1143, 1069, 833, 719; EIMS *m/z* (rel. int.): 252 (100), 237 (81), 224 (52), 209 (62), 189 (20), 177 (11), 149 (12), 139 (12), 93 (13), 77 (28); ^{1}H NMR ($CDCl_3$): 6.84 (1H, *s*, H-8), 6.23 (1H, *br s*, OH-7), 5.94 (1H, *s*, H-3), 3.77 (3H, *s*, OMe-6), 2.77 (3H, *s*, Me-5), 2.50 (3H, *s*, SMe); ^{13}C NMR ($CDCl_3$): 160.1 (C-4), 159.3 (C-2), 151.9 (C-7), 151.5 (C-8a), 143.1 (C-6), 129.6 (C-5), 111.9 (C-4a), 104.3 (C-3), 102.2 (C-8), 61.4 (OMe-6), 16.4 (SMe-4), 16.0 (Me-5); the latter two assignments may be reversed, as may those for C-4/C-2, C-7/C-8a and C-3/C-8.

6,7-*Dimethoxy*-5-*methyl*-4-*methylthiocoumarin* (**1j**). Mp 146–147°; λ^{MeOH} nm (log ε): 225 (4.11), 282 (3.92), 310 (3.96), 331 (3.93), 345 (*sh*., 3.8); ν^{KBr} cm^{-1}: 1703, 1596, 1529, 1403, 1350, 1289, 1233, 1197, 1053, 898, 716; EIMS *m/z* (rel. int.): 266 (100), 251 (54), 238 (24), 223 (31), 77 (14); ^{1}H and ^{13}C NMR data are given in Fig. 3; delays in the FLOCK pulse sequence [8] were Δ^1 = 86.5 msec, Δ^2 = 46.5 msec with a relaxation delay of 2.0 sec.

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REFERENCES

1. Waigh, R. D., Zerihun, B. M. and Euerby, M. R. (1990) *Phytochemistry* **29**, 2935.
2. Okogun, J. I., Enyeniki, V. U. and Ekong, D. E. U. (1978) *Tetrahedron* **34**, 1221.
3. Bohlmann, F. and Zdero, C. (1977) *Phytochemistry* **16**, 239.
4. Bevan, C. W. L. and Ekong, D. E. U. (1965) *Chem. Ind. (Lond.)* 383.
5. Venturella, P., Bellino, A. and Piozzi, F. (1974) *Heterocycles* **2**, 345.
6. Joseph-Nathan, P., Hernandez, J. D., Roman, L. U., Garcia, E., Mendoza, V. and Mendoza, S. (1982) *Phytochemistry* **21**, 1129.
7. Gray, A. I. (1981) *Phytochemistry* **20**, 1711.
8. Reynolds, W. F., McLean, S., Perpick-Dumont, M. and Enriquez, R. G. (1989) *Magn. Reson. Chem.* **27**, 162.
9. Bohlmann, F. and Wienhold, C. (1979) *Chem. Ber.* **112**, 2394.
10. McIntyre, C. R., Reed, D., Sadler, I. H. and Simpson, T. J. (1989) *J. Chem. Soc. Perkin Trans. 1*, 1987.

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CALYSTEGINES IN *SOLANUM* AND *DATURA* SPECIES AND THE DEATH'S-HEAD HAWK-MOTH (*ACHERONTIA ATROPUS*)

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Key Word Index—*Solanum*; *Datura*; Solanaceae; calystegines; tropane alkaloids; glycosidase inhibitors; Lepidoptera; death's-head hawk-moth; ithomiine butterfly.

Abstract—Polyhydroxylated tropane alkaloids, not hitherto recorded from the potato (*Solanum tuberosum*), were detected in the leaves and tubers of this plant. They are potent inhibitors of glycosidases and may be responsible for neurological disorders in livestock. These alkaloids were also extracted from a sphingid moth and an ithomiine butterfly, the larvae of which feed on *Solanum*.

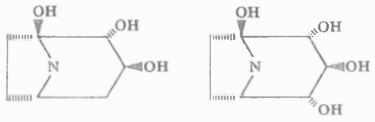
INTRODUCTION

The hawk-moths are essentially a cryptic group both as adult and larva, but among them are a few sequesterers and storers of toxins [1–3] and also a number of enigmatic species which could be classified as either cryptic or aposematic. The death's-head hawk-moth (*Acherontia atropus*), the larvae of which feed on potatoes, pertains to the latter category and its coloration and behaviour suggest that it contains some plant-derived deterrent. We verified that, like *Manduca sexta* and most *Solanum* feeders, this moth does not store the glycoalkaloids present in *Solanum* or the known alkaloids of *Datura*, on which plants we reared it at Ashton. We could, however, detect other unusual bases in several of the moths and therefore decided to search the plants as the possible source of some deterrent hitherto overlooked.

RESULTS AND DISCUSSION

White, healthy tubers of *S. tuberosum* cv 'Estima' were purchased in a supermarket and found to contain 0.01% (fresh weight) of tropane alkaloids in the skin whereas the rest of the tuber contained one-tenth of this concentration. The major alkaloids were 1α,2β,3α-trihydroxy-*nor*-tropane (**1**) and 1α,2β,3α,4β-tetrahydroxy-*nor*-tropane (**2**), recently reported to occur in *Calystegia sepium* (bindweed) and given the trivial names calystegine A_3 and B_2, respectively [4, 5]. The two alkaloids occurred in a 1:2 ratio.

Calystegine B_2 (**2**) was also identified by GC-mass spectrometry in leaves of *S. tuberosum*, cvs 'King Edward', 'Dunluce', 'Desiree' and 'Cara', *Solanum dulcamara* (bittersweet), *Solanum melongena* (aubergine fruits), and in herbarium (R. B. G. Kew) leaf fragments of two species of *Solanum* reported to cause a degenerative neurological disorder in cattle [6]. These are *S. dimidiatum* from Texas (specimen from McLennan County 1946, C. L. York, coll. no. 46113) and *S. kwebense* from Southern Africa (specimen from Namibia 1914, Giess & Miller, coll. no. 11808). *Datura wrightii* leaves also contained calystegine B_2.



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Polyhydroxylated tropane alkaloids have not been previously reported from *Solanum* or *Datura* species and further investigation is necessary before one can claim a defensive function for these substances or possibly a synergistic role associated with the other alkaloids. Nevertheless, it may be significant that *S. dimidiatum* and *S. kwebense* cause the neurological disorders known as 'crazy cow syndrome' and 'Maldronksiekte', respectively, if fed to cattle [6]. The diseases are characterized by depression and muscular incoordination; clinical signs are intense cellular vacuolation and lesions in the cerebellar cortex and degeneration of Purkinje cell axons. They are strikingly similar to locoism, which is caused by ingestion of legumes containing the 1,2,8-trihydroxyindolizidine alkaloid swainsonine [7]. Swainsonine is a potent inhibitor of α-mannosidase and causes vacuolation of CNS cells with neuroaxonal dystrophy

by inhibiting glycoprotein synthesis and degradation [8]. It is interesting that neither tropane alkaloid was strongly inhibitory to jack bean α-mannosidase at 0.3 mM but calystegine B_2 caused 50% inhibition of almond β-glucosidase at 15 μM (35 μM for calystegine A_3). Deficiency of lysosomal β-glucosidase activity is known to cause a glucocerebroside storage disorder with neuronal damage [9]. Yeast α-glucosidase was not inhibited by 0.3 mM of either compound.

It should also be noted that in some cases of 'poisoning' in man and livestock for which sprouted green or spoiled potatoes were held responsible, the concentration of glycoalkaloids was thought to be too low to cause intoxication [10]. Again an unidentified substance in certain tubers has been suggested as a possible cause of anencephaly and spina bifida [11]. It has also been reported (unpublished observations) that diarrhoea in canaries followed the accidental ingestion of 7 mg of a mixture of the two calystegines dissolved in approximately 20 ml of drinking water.

The strain of *Acherontia* used in these experiments was captured in the Canary Isles, feeding on *Datura*, but was subsequently reared on privet (*Ligustrum vulgare*) for a number of generations. They appeared to be no longer well adapted to feed on these natural food plants, for despite great care only a single specimen survived to pupate on *Datura* and the caterpillars reared on *Solanum tuberosum* (cv 'Dunluce') were flaccid, inactive, their faeces liquid, like pale greenish water, and mortality was high—a great contrast to those fed on privet. They lacked glycoalkaloids [2] but contained the glycosidase-inhibiting hydroxylated tropane alkaloids in both pupae and adults. The concentrations present varied greatly from moth to moth.

We also examined dried specimens of the butterfly *Mechanitis polymnia* which feeds in the larval stage on *Solanum*. Keith Brown, in a series of brilliant investigations [12, 13], has shown that this butterfly and related species also do not sequester glycoalkaloids from their food plant, but after eclosion search for, and imbibe, pyrrolizidine alkaloids from the nectar of certain plants, such as *Eupatorium*, and the exudate from specific wilting foliage, which affords them protection from various predators.

We surmised that these butterflies must possess some defence mechanism which protects them during their vulnerable 'searching period' following eclosion. We therefore looked for the tropane alkaloids which might fulfil this role, and found we were able to detect (by GC-mass spectrometry) calystegines in dried specimens collected in 1907.

Where the Lepidoptera are concerned, it is highly significant that the ultra brilliant day-flying moth *Urania fulgens* stores alkaloid glycosidase inhibitors, sequestered from its food plant (Euphorbiaceae) [14]. It is now evident that these compounds are common in various plants. Their storage by certain apparently unprotected, brightly coloured, conspicuous Lepidoptera may be the solution of a perennial puzzle.

Swynnerton [15] who, in Africa, fed literally thousands of insect prey to both wild and captive predators, concluded 'indigestability is the real defence'.

EXPERIMENTAL

Isolation. Plant and insect material was extracted in 75% EtOH (2 × 2 ml 200 mg^{-1}) and the extracts combined. The calystegines were purified by ion exchange chromatography. Calystegine A_3 (**1**) was eluted after calystegine B_2 (**2**) from Amberlite CG-50 (in the H^+ form) using 0.1 M HOAc. The calystegines can be detected by high voltage paper electrophoresis using ninhydrin (0.2% in Me_2CO) with which they give a yellow product, or by GC-MS of the pertrimethylsilyl derivatives (M^+ ions 376 and 464 for A_3 and B_2, respectively). The calystegines are insoluble in $CHCl_3$ and do not react with Dragendorff's or iodoplatinate reagents. ^1H and ^{13}C NMR spectral data matched reported data for the two calystegines [4, 5], but the absolute stereochemistry could not be assigned. EIMS M^+ *m/z* 160 (A_3) and 176 (B_2). Enzyme dosage: all enzyme assays were carried out using enzymes from Sigma (10 μg ml^{-1}) and 10 mM *p*-nitrophenylglycosides [16].

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REFERENCES

1. Marsh, N., Rothschild, M. and Evans, F. (1984) in *The Biology of Butterflies* (Vane-Wright, R. I. and Ackery, P. R., eds), pp. 135–139. Academic Press, London.
2. Rothschild, M. (1985) in *Moths and Butterflies of Great Britain and Ireland*, Vol. 2, pp. 25–31. Harley Books, Colchester.
3. Rothschild, M., Aplin, R., Baker, J. and Marsh, N. (1979) *Nature, Lond.* **280**, 487.
4. Goldmann, A., Milat, M. L., Ducrot, P. H., Lallemand, J. Y., Maillee, M., Lepingle, A., Charpin, I. and Tepfer, D. (1990) *Phytochemistry* **29**, 2125.
5. Duclos, O., Dureault, A. and Depezay, J. C. (1992) *Tetrahedron Letters* **33**, 1059.
6. Menzies, J. S., Bridges, C. H. and Bailey, E. M. (1979) *Southwestern Vet.* **32**, 45.
7. Molyneux, R. J. and James, L. F. (1982) *Science* **216**, 190.
8. Dorling, P. R., Huxtable, C. R. and Vogel, P. (1978) *Neuropathol. Appl. Neurobiol.* **4**, 285.
9. Dorling, P. R. (1984) in *Lysosomes in Biology and Pathology* (Dingle, J. T., Dean, R. T. and Sly, D. W., eds), pp. 347–379. Elsevier, Amsterdam.
10. Baker, D. C., Keeler, R. F. and Gaffield, W. (1991) in *Toxicology of Plant and Fungal Compounds* (Keeler, R. F. and Tu, A. T., eds), pp. 71–82. Dekker, New York.

11. Renwick, J. H. (1972) *Br. J. Prev. Soc. Med.* **26**, 67.
12. Brown, K. S. (1987) *Ann. Missouri Bot. Gard.* **74**, 359.
13. Brown, K. S. and Henriques, S. A. (1991) in *Solanaceae III* (Hawkes, J. G., Lester, R. N., Nee, M. and Estrada, N., eds), pp. 51–68. Royal Botanic Gardens, Kew and Linnean Society of London.
14. Kite, G. C., Horn, J. M., Romeo, J. T., Fellows, L. E., Lees, D. C., Scofield, A. M. and Smith, N. G. (1990) *Phytochemistry* **29**, 103.
15. Swynnerton, C. F. M. (1919) *J. Linn. Soc.* **33**, 203.
16. Saul, R., Chambers, J. P., Molyneux, R. J. and Elbein, A. D. (1983) *Arch. Biochem. Biophys.* **221**, 593.

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A XANTHINE ALKALOID FROM THE LEAVES OF *BOSISTOA FLOYDII*

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Key Word Index—*Bosistoa floydii*; Rutaceae; xanthine alkaloid; 1,3-di(3-methylbut-2-enyl)-7-methylxanthine; flavonoids; NMR-HMBC.

Abstract—A novel xanthine alkaloid and two known flavonoids have been isolated from the leaves of *Bosistoa floydii* and identified as 1,3-di(3-methylbut-2-enyl)-7-methylxanthine, 5,4′-dihydroxy-3,7,3′-trimethoxyflavone and 5-hydroxy-3,3′,4′,5′,7-pentamethoxyflavone.

INTRODUCTION

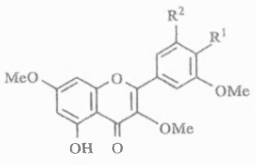
The genus *Bosistoa* F. Muell. *ex* Benth (Rutaceae) is confined to the forests of eastern Australia. *Bosistoa floydii* T. Hartley is a small tree found in rain forest in northeast New South Wales, in the vicinity of Dorigo and Coffs Harbour [1]. No previous chemical work has been carried out on this species and all that is known about the chemistry of the genus is the occurrence of triterpenes in *B. pentacocca* [2] and triterpenes and flavonoids in *B. brassii* [3]. In this paper, we report the result of examination of the leaf of *B. floydii*, with the isolation and identification of a novel xanthine alkaloid and two known flavonoids.

RESULTS AND DISCUSSION

The ground leaves of *B. floydii* were extracted with chloroform and the extract was subjected to VLC over silica gel. Compounds **1**–**3** were purified by preparative circular chromatography and preparative TLC.

Compound **1** was found to be a novel alkaloid, identified as 1,3-di(3-methylbut-2-enyl)-7-methylxanthine on the basis of the following evidence. HREIMS indicated the molecular formula to be $C_{16}H_{22}N_4O_2$. The fragment ion at 166 (80%; $[M-C_{10}H_{16}]^+$) suggested the loss of two isoprene units, and left a skeleton of $C_6H_6N_4O_2$. UV and IR spectra (see Experimental) showed characteristics typical of a xanthine [4, 5]. The ^{1}H NMR spectrum showed signals for two 3-methylbut-2-enyl groups, a methyl singlet at δ3.97 and a proton singlet at δ7.48. The NMR assignments for the compound were made unambiguously by HMBC [6] and H-C COBI experiments (Fig. 1). The H-1′ of one prenyl group showed ^3J coupling with the xanthine carbonyls at C-2 and C-6, while the H-1″ of the other prenyl showed ^3J coupling with C-2 and C-4, so that the prenyl units must be placed at N-1 and N-3. The protons of the N-Me group showed ^3J coupling with C-5 and C-8, while the proton at C-8 showed ^3J coupling with NMe-7, C-5 and C-4. Only structure **1** satisfies all these HMBC correlations.

The two flavonols were identified as 5,4′-dihydroxy-3,7,3′-trimethoxyflavone (pachypodol) (**2**) [7, 8] and 5-hydroxy-3,3′,4′,5′,7-pentamethoxyflavone (combretol) (**3**) [9] by direct comparison of their spectral data with those of authentic samples.



	R^1	R^2
(2)	OH	H
(3)	OMe	OMe

EXPERIMENTAL

UV: 0.1 M HOAc and MeOH; IR: KBr discs; NMR: run in $CDCl_3$, NOESY and HMBC experiments were

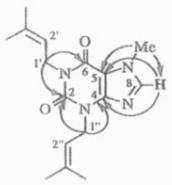


Fig. 1. Selected HMBC correlations for **1**.

obtained on a Bruker AMX-400 instrument using standard microprograms. EIMS: direct probe insert (90–130°) at 70 eV. Petrol refers to bp 60–80° fraction.

Plant material. A voucher (T. G. Hartley 15156) has been deposited at the Australian National Herbarium, Canberra.

Extraction and isolation. The ground leaves (280 g) were extracted sequentially in a Soxhlet with $CHCl_3$ and MeOH. The $CHCl_3$ extract (6 g) was fractionated by VLC over silica gel eluting with petrol containing increasing amounts of EtOAc. A 40–50% EtOAc eluate was collected and the pigments removed by using a short column of Sephadex LH-20. Further fractionation of the sample by circular prep. TLC using petrol–EtOAc followed by prep. TLC (silica gel; $CHCl_3$–EtOAc, 3:2) gave 3 major compounds **1** (200 mg, R_f 0.31), **2** (12 mg, R_f 0.45) and **3** (8.0 mg, R_f 0.55).

1,3-*Di*(3-*methylbut*-2-*enyl*)-7-*methylxanthine* (**1**). Powder. Found: $[M]^+$ 302.1726; $C_{16}H_{22}N_4O_2$ requires 302.1743. UV λ_{max}^{HOAc} nm (log ε): 275 (3.78). IR ν_{max} cm^{-1}: 2900, 1710, 1543, 1510, 1025; ^1H NMR (400 MHz, $CDCl_3$): δ 7.48 (1H, *s*, H-8), 5.35 (1H, *t*, *m*, $J = 6.8$, 1.4 Hz, H-2″), 5.25 (1H, *t*, *m*, $J = 6.8$, 1.4 Hz, H-2′), 4.68 (2H, *d*, $J = 6.7$ Hz, CH_2-1″), 4.60 (2H, *d*, $J = 6.7$ Hz, CH_2-1′), 3.97 (3H, *s*, NMe-7), 1.84 (3H, *s*, Me-5″), 1.80 (3H, *s*, Me-5′), 1.71 (2 × 3H, 2 × *s*, Me-4′, Me-4″); ^{13}C NMR (100 MHz, $CDCl_3$): δ 155.5 (C-6), 151.3 (C-2), 148.6 (C-4), 141.5 (C-8), 137 (C-3″), 136.5 (C-3′), 119.5 (C-2′), 118.8 (C-2″), 108.1 (C-5), 41.6 (C-1″), 39.7 (C-1′), 33.7 (C-NMe-7), 25.9 (C-4″), 25.9 (C-4′), 18.4 (C-5″), 18.3 (C-5′). EIMS *m/z* (rel. int.): 302 $[M]^+$ (65), 234 (38), 166 (80), 150 (100).

5,4′-*Dihydroxy*-3,7,3′-*trimethoxyflavone* (**2**). Yellow needles from $CHCl_3$–MeOH, mp 172° (lit. [8] 168–170°). Found: $[M]^+$ 344.0896; $C_{18}H_{16}O_7$ requires 344.0896. UV λ_{max}^{MeOH} nm: 262, 345. The spectral data are identical with authentic sample.

5-*Hydroxy*-3,3′,4′,5′,7-*pentamethoxyflavone* (**3**). Yellow needles from $CHCl_3$–MeOH, mp 145–150° (lit. [9] 144°). Found: $[M]^+$ 388.1158; $C_{20}H_{20}O_8$ requires 388.1158. UV λ_{max}^{MeOH} nm: 258, 352. The spectral data are identical with authentic sample.

REFERENCES

1. Hartley, T. G. (1977) *J. Arnold Arbor* **58**, 416.
2. Croft, J. A., Ritchie, E. and Taylor, W. C. (1975) *Aust. J. Chem.* **28**, 2093.
3. Parsons, I. C., Gray, A. I. and Waterman, P. G. (1991) *J. Nat. Prod.* **56**, 46.
4. Leonard, N. J. and Blout, E. R. (1950) *J. Chem. Soc.* **72**, 484.
5. Blout, E. R. and Field, M. (1950) *J. Chem. Soc.* **72**, 4880.
6. Bax, A. and Summers, M. F. (1986) *J. Am. Chem. Soc.* **108**, 2093.
7. Valesi, A. G., Rodriguez, E., Veide, G. V. and Mabry, T. J. (1972) *Phytochemistry* **11**, 2821.
8. Fraser, A. W. and Lewis, J. R. (1973) *Phytochemistry* **12**, 1787.
9. Mangkolsuk, S., Dean, F. M. and Houghton, L. E. (1966) *J. Chem. Soc.* (*C*) 125.

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Casuarine: A Very Highly Oxygenated Pyrrolizidine Alkaloid

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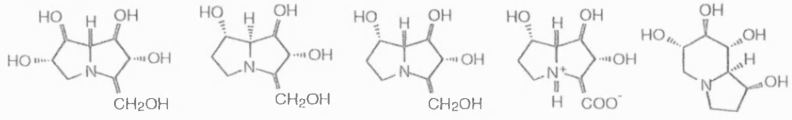
Abstract: The isolation from *Casuarina equisetifolia* L. (Casuarinaceae) bark of casuarine 1, (1R,2R,3R,6S,7S,7aR)-3-(hydroxymethyl)-1,2,6,7-tetrahydroxypyrrolizidine is reported.

Both natural[1] and synthetic mono- and bi-cyclic nitrogen analogues of carbohydrates have potential as chemotherapeutic agents,[2] both as free bases[3] and as alkaloidal glycosides.[4,5] Alexine 2[6] and australine 3[7] were the first pyrrolizidine alkaloids to be isolated with a carbon substituent at C-3, rather than the more usual C-1 substituents.[8] Further stereoisomers of alexine and the related amino acid 7a-epialexaflorine 4, found in *Alexa grandiflora*.,[9] have also been isolated The alexines and castanospermine 5 occur in all species of the genus *Alexa* and also in the related species *Castanospermum australe*; 5 is a potent glucosidase inhibitor[10] some of its derivatives may have potential for the treatment of patients with HIV.[11] Because of the reported antiviral properties of some of the alexines[12,13] and other potential applications,[14] there has been considerable interest in the synthesis of the natural products and of synthetic analogues.[15]



1 2 3 4 5

As part of a programme for the extraction of bioactive compounds from plants, this paper reports the isolation and characterisation of casuarine 1, a more highly oxygenated analogue of 2 and 3 and at the highest oxidation level of any aminosugar analogue yet found as a natural product from any source.

Casuarina equisetifolia L. (Casuarinaceae) wood, bark and leaves have been claimed to be useful against diarrhoea, dysentery and colic.[16] A sample of bark has recently been prescribed in Western Samoa for the treatment of breast cancer; analysis by GC-MS of the pertrimethylsilylated extract of the bark revealed the presence of a pentahydroxylated pyrrolizidine alkaloid and a glycoside thereof as the major nitrogen-containing compounds present. The alkaloids were readily isolated from 75% aqueous ethanol extracts of the bark by ion-exchange chromatography using Amberlite CG120 (NH_4^+ form) by elution with 0.1 M ammonium hydroxide to give first a glycoside of casuarine and subsequently the free casuarine 1, m.p. 181-182°C (from 95% aqueous alcohol), $[\alpha]_D^{24}$ +16.9 (c 0.8 in H_2O),[17] in 0.013% yield; the casuarine glycoside[18] was present at approximately the same concentration as the free base.

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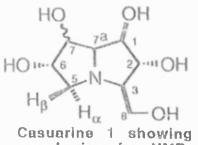

Casuarine 1 showing numbering for NMR figure and table

The ^1H NMR spectrum of casuarine 1 [Figure 1] identified[19] ten inequivalent, non-exchanging protons and eight carbons. The proton chemical shifts, multiplicities and three-bond coupling constants ($^3J_{HH}$) [from the 1D and 2D ^1H-^1H COSY spectra] and carbon chemical shifts, multiplicities and one-bond coupling constants ($^1J_{CH}$) [from the 1D and 2D ^1H-^{13}C HMQC spectra] are given in the table.

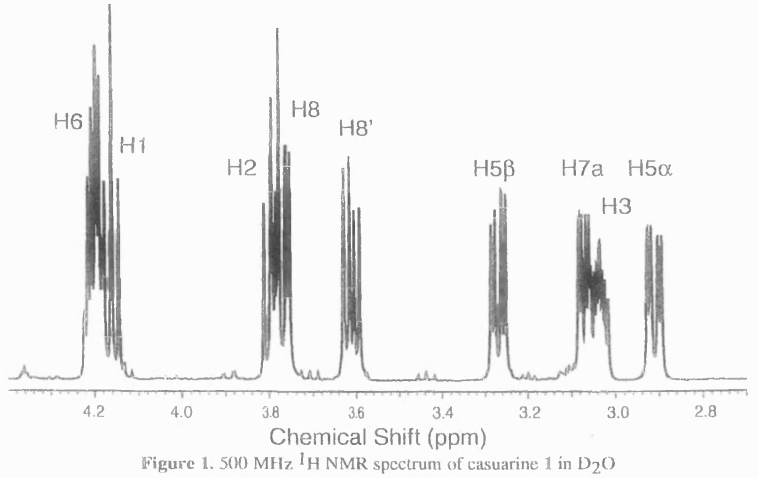

Figure 1. 500 MHz ^1H NMR spectrum of casuarine 1 in D_2O

The 2D COSY and RELAY spectra show a linear sequence of $^3J_{HH}$ correlations as follows: H5α/β -- H6 -- H7 -- H7a -- H1 -- H2 -- H3 -- H8/8' which define the carbon backbone structure. This sequence is confirmed by the $^2J_{CH}$ and $^3J_{CH}$ correlations observed in the HMBC spectrum. In addition, extra $^3J_{CH}$ correlations are observed in the HMBC spectrum as follows: C3 to H5α, H5β and H7a, C5 to H7a and possibly H3, and C7a to H5α and H5β. These define the ring structure as shown above and this is again confirmed by a ^1H-^1H NOE between H3 and H5α. Information about the relative configurations of the 6 chiral carbons may be obtained from the values of the $^3J_{HH}$ coupling constants (related to torsion angles) and ^1H-^1H NOEs (related to distances), if the ring conformations (puckering) can also be determined. The coupling constants of 8.0 Hz for H3/H2, H2/H1 and H1/H7a are only consistent with these four protons being approximately *anti* periplanar, thus defining both the relative configurations at, and ring conformation for, the ring containing these four carbons. Further confirmation of this geometry was obtained by the observation of a stronger NOE between H1 and H3 than between H1 and H2, indicating that H1 and H3 are on the same side of the ring, whilst H2 is on the opposite side. The observation of a strong NOE (corresponding to a distance of 2.5 Å or less) between H3 and H5α and no NOE between H3 and H5β indicates that H3 and H5α must be on the same side of the molecule.

Label	^{1}H			Label	^{13}C		
	δ (ppm)	mult	$^3J_{HH}$ (Hz)		δ (ppm)	mult	$^1J_{CH}$ (Hz)
H1	4.162	t	8.0	C1	77.77	d	146
H2	3.796	t	8.0	C2	76.63	d	139
H3	3.036	m	8.0, 3.8, 6.6	C3	69.97	d	139
H5α	2.911	dd	12.2, 4.0	C5	57.96	t	139
H5β	3.270	dd	12.2, 4.7				
H6	4.21	m	4.0, 4.7, x	C6	77.40	d	151
H7	4.19	m	x, 3.5	C7	78.79	d	148
H7a	3.071	dd	3.5, 8.0	C7a	72.09	d	146
H8	3.771	dd	11.9, 3.8	C8	62.24	t	142
H8'	3.611	dd	11.9, 6.6				

Table ^{1}H and ^{13}C assignments and coupling constants for casuarine in D_2O, pH=8.25 and 30ºC.

A stronger NOE is observed between H6 and H5β than between H6 and H5α, indicating that H6 and H5α are on the same side of the ring. This tentatively assigns the configuration at C6 relative to C3. The coupling constant of 3.5 Hz for H7a/H7 is consistent with either a *cis* or *trans* relationship, depending on the conformation of the second ring. The H7/H6 coupling constant cannot be determined because of considerable overlap in the spectra. Thus NMR studies gave firm indications of the relative configurations at 5 of the chiral centres of casuarine 1 but could not unambiguously assign the relative configuration of the sixth stereogenic centre; an X-ray crystallographic study[20] resolved the ambiguity of the remaining centre and determined the absolute configuration of the new alkaloid (Figure 2).

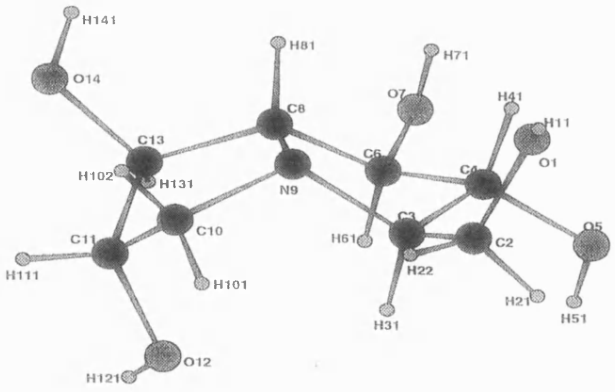

Figure 2. X-ray Molecular Structure of casuarine 1, showing crystallographic numbering scheme

In summary this paper reports the isolation of casuarine, the first example of a pentahydroxylated pyrrolizidine alkaloid with 6 adjacent stereogenic centres and functional groups on all of the 8 carbon atoms, and a structure that provides a considerable challenge for its synthesis.[21]

REFERENCES

[1]Fellows, L. E., Kite, G. C., Nash, R. J., Simmonds, M. S. J., in Poulson, J. E., Romeo, J. J., Conn, E. E. (Eds.) *Nitrogen Metabolism of Plants*, Clarendon Press, Oxford, p.271, **1992**.

[2]Winchester, B., Fleet, G. W. J., *Glycobiology*, 1992, **2**, 199.

[3]Fischl, M. A., Resnick, L., Coombs, R., Kremer, A. B., Pottage, J. C., Fass, R. J., Fife, K. H., Powderly, W. G., Collier, A. C., Aspinall, R. L., Smith, S. I., Kowalski, K. G., Wallemark, C.-B., *J. Acquired Immune Deficiency Syndrome*, 1994, **7**, 139.

[4]Hiraizum, S., Spohr, U., Spiro, R. G., *J. Biol. Chem.*, 1993, **268**, 9927; Ardron, H., Butters, T. D., Platt, F. M., Wormald, M. R., Dwek, R. A., Fleet, G. W. J., Jacob, G. S., *Tetrahedron: Asymm.*, 1993, 4, 2011.

[5]Asano, N., Oseki, K., Kaneko, E., Matsui, K., *Carbohydr. Res.*, 1994, **258**, 255.

[6]Nash, R. J., Fellows, L. E., Dring, J. V., Fleet, G. W. J., Derome, A. E., Hamor, T., Hegarty, M. P., Scofield, A. M., and Watkin, D. J., *Tetrahedron. Lett.*, 1988, **29**, 2487.

[7]R. J. Molyneux, M. Benson, R. Y. Wong, J. E. Tropea, and A. D. Elbein, *J. Nat. Prod.*, 1988, **51**, 1198; C. M. Harris, T. M. Harris, R. J. Molyneux, J. E. Tropea and A. D. Elbein, *Tetrahedron Lett.*, 1989, **30**, 5685.

[8]D. J. Robins, *Nat. Prod. Rep.*, 1990, **7**, 377.

[9]Pereira, A. C. de S., Kaplan, M. A. C., Maia, J. G. S., Gottlieb, O. R., Nash, R. J., Fleet, G., Pearce, L., Watkin, D. J., Scofield, A. M., *Tetrahedron Lett.*, 1991, **47**, 5637.

[10]Nash, R. J., Fellows, L. E., Girdhar, A., Fleet, G. W. J., Peach, J. M., Watkin, D. J., Hegarty, M. P., *Phytochemistry*, 1990, **29**, 1356.

[11]Sunkara, P. S., Taylor, D. L., Kang, M. S., Bowlin, T. L., Liu, P. S., Tyms, A. S., Sjoerdsma, A., *Lancet*, 1989, 1206; Rhinehart, B. L., Robinson, K. M., King, C. R., Liu, P. S., *Biochem. Pharmacol.*, 1990, **39**, 1537.

[12]Fellows, L., Nash, R., *Chem. Abs.*, 1990, **114**, 143777s; Elbein, A. D., Tropea, J. E., Molyneux, R. J., *Chem. Abs.*, 1989, **113**, 91444p.

[13]Taylor, D. L., Nash, R., Fellows, L. E., Kang, M. S., Tyms, A. S., *Antiviral Chem. Chemother.*, 1992, **3**, 273.

[14]Molyneux, R. J., Pan, Y. T., Tropea, Joseph E., Benson, M., Kaushal, G. P., Elbein, Alan D., *Biochemistry*, 1990, **30**, 9981; Simmonds, M. S. J., Blaney, W. M., Fellows, L. E., *J. Chem. Ecol.*, 1990, **16**, 3167.

[15]Fleet, G. W. J., Haraldsson, M., Nash, R. J., Fellows, L. E., *Tetrahedron Lett.*, 1988, **29**, 5441; Pearson, W. H., Hines, J. V., *Tetrahedron Lett.*, 1991, **32**, 5513; Frankowski, A., Seliga, C., Bur, D., Streith, J., *Helv. Chim. Acta*, 1991, **74**, 934; Choi, S., Bruce, I., Fairbanks, A. J., Fleet, G. W., Jones, A. H., Nash, R. J., Fellows, L. E., *Tetrahedron Lett.*, 1991, **32**, 5517; Ikota, N., *Tetrahedron Lett.*, 1992, **33**, 2553.

[16]Chopra, R.N., Nayar, S. L., Chopra, I. C., *Glossary of Indian Medicinal Plants*, Council of Scientific and Industrial Research (India), New Delhi, **1956**, p. 55.

[17] For casuarine 1 - found: C, 46.59; H, 7.75; N, 6.62%. $C_8H_{15}NO_5$ requires C, 46.82; H, 7.37; N, 6.83%.

[18]The structure of the casuarine glycoside is currently under investigation.

[19]NMR abbreviations: COSY - correlation spectroscopy; HMBC - heteronuclear multiple bond correlation spectroscopy; HMQC - heteronuclear multiple quantum correlation spectroscopy; NMR - nuclear magnetic resonance; NOE - nuclear Overhauser effect; NOESY - nuclear Overhauser effect spectroscopy; RELAY - relayed correlation spectroscopy. All NMR spectra were recorded on a Varian Unity 500 spectrometer, with a probe temperature of 30°C at pH 8.25; ^1H and ^{13}C chemical shifts are referenced to trimethylsilylpropanesulphonic acid at δ 0.000and acetone at δ 29.80, respectively. All two-dimensional spectra (^1H-^1H COSY, RELAY and NOESY, ^1H-^{13}C HMQC and HMBC) were acquired in phase-sensitive mode. An 8 Hz relay step was used for the RELAY spectrum and NOESY spectra were recorded with mixing times of 100 msec to 500 msec.

[20]The atomic coordinates for casuarine 1 are available on request from the Cambridge Crystallographic Data Centre, University Chemistry Laboratory, Lensfield Road, Cambridge CB2 1EW; the crystallographic numbering system differs from that used for 1 elsewhere in the text. Any requests should be accompanied by the full literature citation for this paper.

[21]We thank Miss Marianne Sims and Mr Richard Layton for excellent technical assistance (IGER).

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Potent Antimalarial Activity of the Alkaloid Nitidine, Isolated from a Kenyan Herbal Remedy

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Bioassay-guided fractionation of extracts of *Toddalia asiatica*, a plant used by the Pokot tribe of Kenya to treat fevers, has yielded the alkaloid nitidine as the major antimalarial component. Fractions containing nitidine have in vitro 50% inhibitory concentrations against *Plasmodium falciparum* in the range of 9 to 108 ng/ml for a range of chloroquine-susceptible and -resistant strains. The results show a lack of cross-resistance between chloroquine and nitidine.

Although malaria is in principle preventable and curable, in practice the majority of the population at risk cannot pay for modern treatment and the disease remains a major cause of childhood mortality in the developing world. Recent results with the vaccine sPf66 show a mean protective effect of only 30%, with very wide confidence limits (1). The rapid spread of resistance to chloroquine and related quinoline-based antimalarial agents has greatly increased the risk of malaria to many rural populations, so that there is an urgent need for affordable treatment. One possible source of such treatment lies in the traditional herbal remedies used by ethnic groups, but treatment with these remedies has suffered from a number of deficiencies. Diagnosis is often a problem, identification of plant material may be insecure, and the chemical content of extracts may vary considerably. We have set out to overcome these barriers to effective treatment by systematically evaluating the effectiveness of some of the remedies used by an ethnic group of Kenyan farmers.

The Pokot traditionally inhabit a highland plateau, west of the Rift Valley in Kenya. However, the present study concerned a group of Pokot who live on Ol Ari Nyiro Ranch, Laikipia, Kenya, a plateau to the east of the Rift Valley, and who have previously been described in some detail (5).

In interviews, the Pokot herbalist Cheptosai Selale, who is 90 years old, described the use of 26 plants for the treatment of malaria and fever, of which only 14 were available at the time of collection (see Table 1). Two of the 14 plants produced extracts with significant antimalarial activity against laboratory strains of *Plasmodium falciparum*. Bioassay-guided fractionation of the extract from *Toddalia asiatica* gave a pure alkaloid, nitidine, which has potentially useful antimalarial activity.

MATERIALS AND METHODS

Plant material. Plant material was collected from Ol Ari Nyiro Ranch and was botanically authenticated by Christine Kabuye and Joshua Muasya of the Herbarium, National Museums of Kenya, where voucher specimens were deposited. Information provided by the herbalist included the required part of the plant, the precise locality for collection, and the time when curative potency was maximal. Plant material for study was dried at room temperature, pulverized, and stored dry in plastic bags until extracts were obtained.

Preparation of aqueous crude extracts for preliminary analysis. Aqueous crude extracts (10%) were prepared in a manner analogous to that used by the herbalist. Powdered plant material (10 g) was weighed into a beaker, 100 ml of distilled water was added, and the mixture was brought to the boil, cooled, and left overnight to macerate. Extracts were filtered through no. 1 Whatman filter paper, and the volume of filtrate was noted. Solutions were then sequentially filtered through membrane filters (Millipore, Harrow, United Kingdom) with pore sizes of 0.8, 0.45, and finally, 0.22 μm, and the final volume of filtrate was noted. Aliquots of 2 ml were freeze-dried to determine the concentration (in grams per milliliter) of solute in each extract before freeze-drying the remaining crude extract.

Preparation of extracts in a form suitable for the in vitro test required consideration of both the aqueous solubility and the sterility of each extract. Each compound was redissolved in a measured volume of either water or ethanol, which was then further diluted with its counterpart to yield a 70% ethanol–30% water mixture containing a known concentration of extract. This solution was allowed to stand at room temperature for 30 min to sterilize the solution. Further dilutions were made with *P. falciparum* culture medium (RPMI 1640 medium containing 25 mmol of HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] buffer per liter, 25 mmol of sodium bicarbonate per liter, and 10% normal, pooled human serum). Final dilutions on the in vitro test plate contained <0.1% ethanol, which is not inhibitory to the parasite in this system (3), although controls were included in each test series.

Extraction and fractionation of *T. asiatica*. Air-dried, pulverized root bark was first extracted twice with dichloromethane (500 g to 1 liter) by overnight maceration to remove fats. The residue was then extracted twice with cold methanol (500 g to 1 liter), and the filtrates were combined and dried in vacuo. The alkaloids in the crude methanolic fraction were concentrated by the acid-base procedure (6), and the resulting chloroform fraction was further fractionated by vacuum-liquid column chromatography with silica gel 60 and a solvent system of increasing polarity (petrol, chloroform, methanol). Fifteen fractions were collected, dried in vacuo, and stored at 4°C until they were tested.

For in vitro tests, approximately 10 mg, weighed to 0.01 mg, was dissolved in 70% ethanol and was allowed to stand for 30 min to sterilize the solution. The ethanolic solution was further diluted in culture medium to an appropriate test concentration. A stock solution of authentic nitidine was prepared in a similar manner.

In vitro antimalarial activity tests. The antimalarial activity test was based on previously reported methods (3, 10). Laboratory cultures of *P. falciparum* were maintained by standard methods. For the test, 25-μl aliquots of culture medium were added to all of the wells of a 96-well flat-bottom microculture plate (Sterilin, Teddington, United Kingdom). Aliquots of the test solutions of 25 μl were added, in duplicate, to the first wells, and a Titertek motorized hand diluter (Flow Laboratories, Uxbridge, United Kingdom) was used to make serial 2-fold dilutions of each sample over a 64-fold concentration range. Aliquots of 200 μl of a 1.5% (vol/vol) suspension of parasitized erythrocytes in culture medium (0.4% parasitemia; growth rate, >threefold per 48 h) were added to all test wells. Parasitized and nonparasitized erythrocytes and solvent controls were incorporated into all tests. The plates were incubated at 37°C in a gas mixture of 3% CO_2–5% O_2–92% N_2. After 48 h each well was pulsed with 25 μl of culture medium containing 0.5 μCi of [^{3}H]hypoxanthine and the plates were incubated for a further 18 h. The contents of each well were then harvested onto glass fiber filters, washed thoroughly with distilled water, and dried, and the radioactivity (in counts per minute) was measured by liquid scintillation. The regression function $\log \text{cpm} = a - b \cdot (\log \text{drug concentration})$, where a is the y intercept and b is

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TABLE 1. In vitro activities of aqueous extracts from Kenyan plants used by the Pokot herbalists, Laikipia, Kenya, against three Kenyan strains of *P. falciparum*

Plant species	Family	Plant part	IC_{50} (μg/ml)[a]		
			M24	K67	ENT 7
Canthium phyllanthoideum	*Rubiaceae*	Stem bark	208	360	540
Olinia usambarensis	*Oliniaceae*	Stem bark	204	130	380
Pittosporum vividiflorum	*Pittosporaceae*	Stem bark	80	30	170
Aspilia mossambicensis	*Compositae*	Leaves	406	730	720
Gardenia jovis-tonantis	*Rubiaceae*	Root bark	820	880	1,750
Toddalia asiatica	*Rutaceae*	Root bark	20	5	20
Rhoicissus tridentata	*Vitaceae*	Whole tuber	100	40	70
Turraea mombassana	*Meliaceae*	Whole root	150	90	100
Heteromorpha trifolia	*Umbelliferae*	Stem bark	475	370	580
Shrebera alata	*Oleaceae*	Stem bark	627	160	380
Maytenus arbutifolia	*Celastraceae*	Whole root	4	4	10
Rhamnus staddo	*Rhamnaceae*	Root bark	520	490	740
Scutia myrtina	*Rhamnaceae*	Root bark	40	240	320
Cucumis aculeatus	*Cucurbitaceae*	Whole fruit	60	30	30

[a] Each IC_{50} is the mean of at least two in vitro tests carried out on different days.

the slope, was calculated by using duplicate points above and below the midpoint of the counts per minute between the parasitized and nonparasitized controls. The concentration causing 50% inhibition of radioisotope incorporation (IC_{50}) was determined by interpolation as described previously (13). This method gives a value equal to 100.4% ± 10.6% (mean ± standard deviation) of the value estimated by nonlinear regression analysis (14). Statistical differences between means for small populations were examined by Student's *t* test.

RESULTS

The results of tests with crude extracts against three strains of *P. falciparum* are presented in Table 1. Only the extracts of the root bark of *T. asiatica* and the whole root of *Maytenus arbutifolia* showed significant activity in this test. Although this preliminary screen does not preclude the possibility that the other extracts may be active antimalarial agents by the oral route, subsequent work focused on the plants with demonstrable activity. Of these, *M. arbutifolia* was known as a source of cytotoxic agents such as maytansine (7) and produced extracts of great complexity; the present study therefore concentrated on *T. asiatica*, which has also been intensively studied (2) but which proved to be much more amenable to bioassay-guided fractionation.

Sequential extracts from *T. asiatica* showed different levels of antimalarial activity (Table 2). The methanolic extract showed the highest activity, with a mean 50% inhibitory dose of 0.98 μg/ml for the chloroquine-susceptible strains K67, K39, and M24. Acid-base extraction of the methanolic extract (6.08 g) yielded 0.1893 g (3.1%) of petroleum ether-soluble extract, 0.0246 g (0.4%) of acidic chloroform extract, and 0.99 g (16.3%) of basic chloroform extract. The highest potency was observed in the basic chloroform extract coded TA106(13), which had an IC_{50} of <1 μg/ml. This extract was further fractionated by chromatography on silica by circular preparative thin-layer chromatography by eluting with ethyl acetate-methanol-water-ammonia (100:17:13:3), which gave a single yellow compound at a 10-mg yield from 500 g of powdered root bark. The UV spectrum showed absorbances at 235, 275, 293, 303, 332, and 380 nm, which is similar to the data for nitidine (18). Comparison with an authentic sample of nitidine (compound 1) both chromatographically and spectroscopically (mass spectrometry, infrared spectroscopy, and high-field nuclear magnetic resonance) proved the identity of the unknown compound.

TABLE 2. In vitro activities of extract fractions from *T. asiatica* against *P. falciparum*

Extract	IC_{50} (μg/ml)[a]		
	K67	K39	M24
Dichloromethane	11.93	7.65	18.05
Methanol	0.7	0.78	1.46
Water	21.14	31.67	740.0
Active fraction		0.04	
Nitidine		0.045	
Dihydronitidine		1.03	
Chloroquine		0.004	

[a] Each IC_{50} is the mean of at least two in vitro tests carried out on different days. For the V1/s strain, IC_{50}s were 0.057 μg/ml for chloroquine and 0.941 μg/ml for dihydronitidine.

The electron impact mass spectrum gave a molecular ion at *m/z* 348 with the formula $C_{21}H_{18}NO_4$ by accurate mass measurement. The base peak in the spectrum, at *m/z* 333, is attributable to the loss of a methyl group. The ^1H nuclear magnetic resonance spectrum showed seven aromatic protons, five singlets at δ 9.61, 8.27, 8.22, 7.80, and 7.59, as well as two doublets (J = 9.2 Hz) centered at δ 8.72 and 8.21. The methylenedioxy group resonated as a two-proton singlet at δ 6.27, and there were three signals for methyl groups at δ 4.93, 4.26, and 4.11, with that at the lowest field being attributable to the *N*-methyl. Comparison of the active fraction with an authentic sample of nitidine by thin-layer chromatography on silica with ethyl acetate-methanol-water-ammonia (100:17:13:3) as the mobile phase gave spots with R_f values of 0.25, having the same color and response to UV light.

Subsequent in vitro studies examined the chemosensitivity of additional laboratory strains of *P. falciparum*, characterized for chloroquine susceptibility, to the active fraction and authentic nitidine by using three chloroquine-resistant and four chloroquine-susceptible strains. Each IC_{50} measurement was determined in duplicate as described above, and each test was performed in duplicate; Table 3 compares the activities of the active fraction, authentic nitidine, and chloroquine against groups of chloroquine-susceptible and chloroquine-resistant laboratory strains of *P. falciparum* in vitro.

DISCUSSION

The antimalarial activities of a number of Tanzanian plants, many of which also occur in Kenya, have been reviewed by

TABLE 3. In vitro chemosensitivity of *P. falciparum* isolates to an alkaloidal fraction [TA106(13)] from *T. asiatica*, nitidine, and chloroquine

Isolate	Chloroquine susceptibility	IC_{50} (ng/ml)		
		Chloroquine	TA106(13)	Nitidine
UPA	Susceptible	16.0	56.0	67.0
K39	Susceptible	4.4	40.3	45.1
SL/D6	Susceptible	6.6	29.3	76.0
HB3	Susceptible	5.9	40.4	73.6
		8.22 ± 5.3	41.5 ± 11.0	65.4 ± 14.1
ItD12	Resistant	65.9	9.2	42.0
FCR3	Resistant	47.4	37.5	165
FCB	Resistant	28.4	108.0	47.5
		47.2 ± 15.3	51.6 ± 41.5	84.8 ± 56.7

[a] Each IC_{50} is the mean of at least two in vitro tests carried out on different days.

Weenen et al. (17). Only 2 of 49 plants from that review (*Gardenia jovis-tonantis* and *T. asiatica*) were also used by the Kenyan Pokot herbalists. It is of interest that *T. asiatica*, one of the two plants producing active extracts in our study, is also used by herbalists in Tanzania and has been reported to produce extracts with moderate in vitro activity against the parasite (16).

Nitidine is a well-known cytotoxic agent which has received considerable attention as a potential anticancer drug following the discovery of potent antileukemic activity in mice (9). Clinical trials were eventually terminated for reasons which are unclear but which may have been due to host toxicity. Nitidine is a quaternary salt, charged at all pH values, and therefore, in theory it is prone to poor absorption from the gastrointestinal tract, but it is capable of existing as the tautomeric pseudobase (compound 2), which would be absorbable (Fig. 1). It was also possible that dihydronitidine (compound 3), identified as a minor component of *T. asiatica*, could account for the in vivo antimalarial activity. Dihydronitidine is not charged and is therefore able to pass membranes; inside cells it could be oxidized to release nitidine. However, dihydronitidine was only weakly antimalarial (Table 2), suggesting that it is not a prodrug for nitidine in our test system.

Although the crude aqueous extract, with an IC_{50} of 20 μg/ml, exhibited comparatively weak activity, when we compared both the active fraction of *T. asiatica* and authentic nitidine against *P. falciparum* strains with different responses to chloroquine, it was apparent that the alkaloid exerted potent activity (Table 3). The active fraction, with a mean IC_{50} of 51.6 ng/ml for chloroquine-resistant strains, is more active than quinine against Kenyan parasites (IC_{50}, ca. 120 ng/ml) (11).

The isolation procedure concentrated on obtaining a sample pure enough for identification. No attempt was made to determine the total amount of nitidine in the root bark. Such a determination would be complicated by the known instability of nitidine in alkaline solution, whereby the pseudobase form (compound 2) disproportionates to give the dihydro form (compound 3) and the 6-keto form known as oxynitidine, which is not charged and which is unlikely to be biologically active. Our experience with other species of the family *Rutaceae* suggests that there are unlikely to be major amounts of alkaloids similar to nitidine in the root bark of *T. asiatica*, nor was there chromatographic evidence for other major active constituents. This does not preclude the existence of chemically unrelated compounds in the extracts with direct or synergistic activity; however, from the data in Tables 1 and 2, it may be seen that nitidine is two orders of magnitude more potent than the crude extract. Given the inevitable losses in the extraction process, there is little doubt that nitidine accounts for a high proportion of the observed activity.

The mean IC_{50} of chloroquine for the chloroquine-resistant isolates of 47.2 ng/ml (189 nmol/liter) exceeds the threshold MIC of 114 nmol/liter used to define chloroquine-resistant infections in Kenya and elsewhere (12, 15), confirming the chemosensitivity of the isolates used. We found a significant difference in the mean IC_{50} of chloroquine between those for chloroquine-resistant isolates and those for chloroquine-susceptible isolates ($P < 0.05$) but not between the IC_{50}s of the active fraction or nitidine ($P > 0.05$ for both comparisons). The resistance of *P. falciparum* to chloroquine is now a major health problem in Kenya, as in many parts of Africa, and these data suggest a potential role for *T. asiatica* extracts in the treatment of chloroquine-resistant falciparum malaria. Further work is needed to confirm the activity of this alkaloidal extract against chloroquine-resistant parasites and to determine more

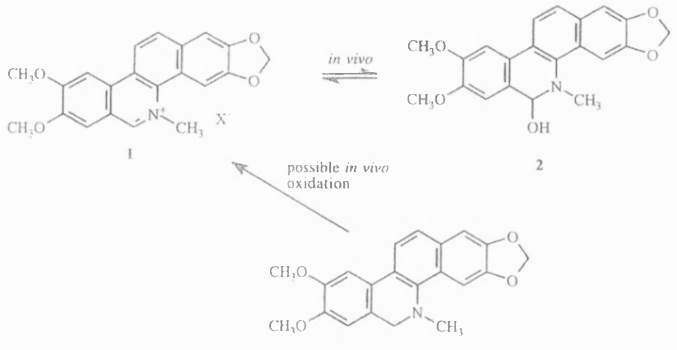

FIG. 1. Chemical structures of nitidine (compound 1), the tautomeric pseudobase form (compound 2), and the possible prodrug 5,6-dihydronitidine (compound 3).

exactly the compositions of the extracts prepared by the Pokot herbalists.

It is possible that long-term consumption of *T. asiatica* may be deleterious, given the cytotoxic effects of nitidine, although short-term use to treat a life-threatening disease may, in future, be supportable on risk-benefit analysis and in view of the availability of the plant and its low cost. Nitidine and its sister alkaloid fagaronine have recently been shown to be topoisomerase inhibitors (4, 8); thus, it is possible that the antimalarial action is mediated through the inhibition of the parasite enzyme. If this is the case, analogs which bind preferentially to the parasite enzyme may represent a source of new antimalarial drugs, and we are pursuing this. The absence of cross-resistance between nitidine-containing extracts and chloroquine may be of particular significance in the potential use of these compounds as drugs for antimalarial treatment.

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REFERENCES

1. **Alonso, P. L., T. Smith, J. R. M. Armstrong-Schellenberg, H. Masanja, S. Mwankusye, H. Urassa, I. De Bastos Azevedo, J. Chongela, S. Kobero, C. Menendez, N. Hurt, M. C. Thomas, E. Lyimo, N. A. Weiss, R. Hayes, A. Y. Kitua, M. C. Lopez, W. L. Kilama, T. Teuscher, and M. Tanner.** 1994. Randomised trial of efficacy of SPf66 vaccine against *Plasmodium falciparum* malaria in children in Southern Tanzania. Lancet **344**:1175–1181.
2. **Chen, I.-S., I.-L. Tsai, S.-J. Wu, W.-S. Sheen, T. Ishikawa, and H. Ishii.** 1993. Toddaquinoline from Formosan *Toddalia asiatica*. Phytochemistry **34**:1449–1451.
3. **Desjardins, R. E., C. J. Canfield, J. D. Haynes, and J. D. Chulay.** 1979. Quantitative assessment of antimalarial activity in vitro by a semiautomated microdilution technique. Antimicrob. Agents Chemother. **16**:710–718.
4. **Fang, S. D., L. K. Wang, and S. M. Hecht.** 1993. Inhibitors of DNA topoisomerase I isolated from the roots of *Zanthoxylum nitidum*. J. Org. Chem. **58**:5025–5027.
5. **Gallman, K.** 1991. I dreamed of Africa. Viking Penguin, New York.
6. **Harborne, J. B.** 1984. Phytochemical methods. 2nd ed. Chapman & Hall, London.
7. **Komoda, Y., and T. Kishi.** 1980. Anticancer agents based on natural products. Academic Press, Inc., New York.
8. **Larsen, A. K., L. Grondard, J. Couprie, B. Desoize, L. Comoe, J. C. Jardillier, and J. R. Riou.** 1993. The antileukaemic alkaloid fagaronine is an inhibitor of DNA topoisomerases I and II. Biochem. Pharmacol. **46**:1403–1412.
9. **Messmer, W. M., M. Tin-Wa, H. H. S. Fong, C. Bevelle, N. R. Farnsworth, D. J. Abraham, and J. Trojanek.** 1972. Fagaronine, a new tumor inhibitor isolated from *Fagara zanthoxyloides* Lam. (Rutaceae). J. Pharm. Sci. **61**:1858.
10. **O'Neill, M. J., D. H. Bray, P. Boardman, J. D. Phillipson, and D. C. Warhurst.** 1985. Plants as sources of antimalarial drugs. Part 1. *In vitro* test method for the evaluation of crude extracts from plants. Planta Medica **50**:394–397.
11. **Pasvol, G., C. R. J. C. Newton, P. A. Winstanley, W. M. Watkins, N. M. Peshu, J. B. O. Were, K. Marsh, and D. A. Warrell.** 1991. Quinine treatment of severe falciparum malaria in African children: randomized comparison of three regimens. Am. J. Trop. Med. Hyg. **45**:702–713.
12. **Rieckman, K. H., L. J. Sax, G. H. Campbell, and J. E. Mrema.** 1978. Drug sensitivity of *Plasmodium falciparum*: an *in vitro* microtechnique. Lancet **311**:22–23.
13. **Sixsmith, D. G., W. M. Watkins, J. D. Chulay, and H. C. Spencer.** 1984. *In vitro* antimalarial activity of tetrahydrofolate dehydrogenase inhibitors. Am. J. Hyg. Trop. Med. **33**:772–776.
14. **Watkins, W. M., D. G. Sixsmith, and J. D. Chulay.** 1984. The activity of proguanil and its metabolites cycloguanil and *p*-chlorophenylbiguanide against *Plasmodium falciparum in vitro*. Ann. Trop. Med. Parasitol. **78**:273–278.
15. **Watkins, W. M., D. G. Sixsmith, H. C. Spencer, D. A. Boriga, D. M. Kuriuki, T. Kipingor, and D. K. Koech.** 1984. Effectiveness of amodiaquine as treatment for chloroquine-resistant *Plasmodium falciparum* infections in Kenya. Lancet **323**:357–359.
16. **Watt, J. M., and M. G. Breyer-Brandwijk.** 1962. Medicinal and poisonous plants of southern and eastern Africa. 2nd ed. Livingstone, Edinburgh.
17. **Weenen, H., M. H. H. Nkunya, D. H. Bray, L. B. Mwasumbi, L. S. Kinabo, and V. Kilimali.** 1990. Antimalarial activity of Tanzanian medicinal plants. Planta Medica **56**:368–370.
18. **Zee-Cheng, K.-Y., and C. C. Cheng.** 1973. Synthesis of 5,6-dihydro-6-methoxynitidine and a practical preparation of nitidine chloride. J. Heterocycl. Chem. **10**:85–88.

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A *SECO*-OLEAN-18-ENE TRITERPENE ACID FROM *VAHLIA CAPENSIS*

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Key Word Index—*Vahlia capensis*; Vahliaceae; triterpene; 3,4 *seco*-olean-4,(23),18 dien-3-oic acid.

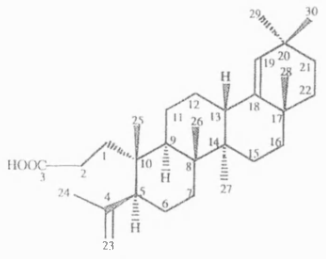
Abstract—The *n*-hexane and ethyl acetate extracts of the aerial parts of *Vahlia capensis* yielded the coumarins umbelliferone, scopoletin and scoparone, and the terpenoids sitosterol, cycloartenol, 24-methylenecycloartanol, cycloart-23-en-3β,25-diol, glutinol and a *seco* ring-A triterpene acid, 3,4-*seco*-olean-4,(23),18-dien-3-oic acid.

INTRODUCTION

The small perennial shrub *Vahlia capensis* (L. f) Thunb. ssp. *vulgaris* Bridson is found mainly in the grazing areas of Botswana, Lesotho, Namibia, Zimbabwe and South Africa [1]. The genus *Vahlia* was initially placed in the family Saxifragaceae, but later moved into a family of its own, the Vahliaceae [1]. *Vahlia capensis* is a medicinal plant that has been used widely in Botswana to cure sore eyes, especially in small children [2]. To date there has been no phytochemical or pharmacological report on the plant.

RESULTS AND DISCUSSION

The compound 3,4 *seco*-olean-4,(23), 18-dien-3-oic acid (1) was obtained from the *n*-hexane and ethyl acetate extracts of powdered aerial parts by a combination of column chromatography and PTLC. The ^{1}H NMR spectrum (Table 1) revealed that 1 contained seven methyl singlets, resonating in the chemical shift range δ_H 0.77–1.75, while a combination of ^{13}C NMR with ^{1}H broad band decoupling, JMOD and DEPT-135 determined both the number and nature of the carbon atoms. The signal at δ_H 1.75 (3H) suggested an olefinic methyl group [3–5] Me-C =], while signals at δ_H 4.70 (1H *br s*) and 4.90 (1H *br s*) indicated an exomethylene group, and a further broad singlet, also at δ_H 4.90, suggested an olefinic proton forming part of a trisubstituted double bond. The IR data (ν_{max} at 1750 and 3560 cm^{-1}) indicated a carboxylic acid, while the absorptions at ν_{max} 1360, 890 and 850 cm^{-1} pointed to the presence of geminal dimethyls, an olefinic CH and an exomethylene ($C=CH_2$) group, respectively.



1

The high resolution EI mass spectrum of the compound gave a molecular ion at *m/z* 440.3654, which solved for the empirical formula $C_{30}H_{48}O_2$. The calculated double bond equivalents are seven which can be accounted for by two double bonds, one carbonyl and four rings. The observed mass spectral fragment at *m/z* 367 for loss of $C_3H_5O_2$ [M − 73]$^+$ is consistent with the presence of a $-CH_2CH_2COOH$ moiety leading to the conclusion that the compound possesses a *seco*-oleanene skeleton. The base peak at *m/z* 177 $[C_{13}H_{21}]^+$ and other high intensity ions at *m/z* 205 $[C_{15}H_{25}]^+$, 204 $[C_{15}H_{24}]^+$ and 189 $[C_{14}H_{21}]^+$ strongly supported a Δ^{18}-oleanene derivative [6]. The latter three ions have been reported in Δ^{18}-oleanenes, e.g. methyl moronate, methyl morolate and germanicol acetate, and arise by a retro-Diels–Alder reaction involving the rupture of ring C followed by loss of a methyl [6]. The ion at *m/z* 177 $[C_{13}H_{21}]^+$, found in those derivatives with a methyl at C-17 (Δ^{18}-oleanene and germanicol acetate), is thought to arise from a fragment at *m/z* 205 by another retro-Diels–Alder reaction involving the elimination of ethene [6].

The ^{1}H and ^{13}C NMR data were very similar to literature values for other Δ^{18}-oleanene derivatives [7–10]. Application of 2D NMR methods (HMBC, HC-COBI and NOESY) allowed the resolution of structure 1 as

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Table 1. ^{1}H–^{1}H COSY and ^{13}C–^{1}H direct for **1**

C	COSY	H	^{13}C–^{1}H direct	C
1		1.64–1.70		34.3 (*t*)
2		2.23–2.26		28.3 (*t*)[a]
		2.36–2.40		
3				180.0 (*s*)
4				157.7 (*s*)
5		1.95–1.98		50.7 (*d*)
6		1.37–1.39		24.8 (*t*)[b]
		1.78–1.81		
7		1.10–1.14		33.6 (*t*)
		1.39–1.41		
8				40.6 (*s*)*
9		1.49–1.50		41.6 (*d*)
10				39.6 (*s*)
11		1.26–1.30		21.6 (*t*)
		1.44–1.48		
12		1.19–1.22		26.3 (*t*)[c]
		1.48–1.50		
13		2.27–2.30 *br d*		38.8 (*d*)
14				43.6 (*s*)*
15		1.78–1.81		27.7 (*t*)
		1.10–1.14		
16		1.34–1.40		37.8 (*t*)*
17				34.6 (*s*)
18				142.8 (*s*)
19		4.90 *br s*		130.1 (*d*)
20				32.6 (*s*)
21		1.44–1.50		33.3 (*t*)
22		1.56–1.50		37.6 (*t*)*
23		4.7 *br s*		113.7 (*t*)
		4.9 *br s*		
24		1.75 3H *s*		23.4 (*q*)
25		0.90 3H *s*		20.9 (*q*)
26		1.13 3H *s*		16.5 (*q*)
27		0.77 3H *s*		14.8 (*q*)
28		1.04 3H *s*		25.5 (*q*)
29		0.95 3H *s*		29.4 (*q*)*
30		0.96 3H *s*		31.6 (*q*)*

* Assignments may be reversed.

[a–c] Assignments which differ from those of Tanaka *et al.* [11].

3,4-*seco*-olean-4(23), 18-dien-3-oic acid which was very recently reported by Tanaka *et al.* [11] from *Euphorbia chamaesyce* (Euphorbiaceae). The NMR data reported for **1** [11] agree with our findings with the exception of the ^{13}C assignments for C-2, C-6 and C-12. The long range heteronuclear coupling data we obtained allowed C-2, C-6 and C-12 to be assigned unambiguously and also agree with published data on related compounds [9]. The melting points also differ significantly, which may be explained by use of different solvents for recrystallization.

The Δ^{12}-isomer of **1**, nyctanthic acid was first isolated from the shrub *Nyctanthis abor-tristis* by Turnbull *et al.* [12, 13]. This acid was later isolated mixed with roburic acid from oak galls formed by the insect *Cynips mayri* on common oak (*Quercus robur*) [14].

EXPERIMENTAL

General. Mp: uncorr. ^{1}H (250, 400 MHz) and ^{13}C (100 MHz) NMR: $CDCl_3$ with TMS as int. standard. HR EIMS: 70 eV. CC: silica gel 60 (70–230 mesh). TLC: Merck silica gel 60 F_{254} (0.25 mm). PTLC: silica gel 60 F_{254} (0.50 mm). Visualization: UV radiation λ-254, 366 nm and vanillin sulphuric acid. The plant material was collected in Serowe, Central District, Botswana, in January 1991 and identified at the National Herbarium, Gaborone, Botswana, where a voucher specimen was deposited.

Extraction and isolation. The whole plant (1 kg) was ground and extracted at room temp. successively with *n*-hexane, EtOAc and MeOH. The *n*-hexane and the EtOAc extracts were evapd *in vacuo* to give 6 and 10 g, respectively, of dark green tar. The combined extracts were subjected to CC using *n*-hexane with increasing proportions of EtOAc. The fr. showing distinctive streaking on analyt. TLC plates, that gave a dark brown band upon spraying with vanillin sulphuric acid, was further purified (PTLC: toluene–EtOAc-HOAc 35:14:1) to give needles of **1** which were recrystallized from hexane (15 mg). Other frs yielded umbelliferone (8 mg), scopoletin (5 mg), scoparone (4 mg), sitosterol (30 mg), cycloartenol (10 mg), cycloart-23-en-3β,35-diol (15 mg), 24-methylenecycloartanol (25 mg) and glutinol (8 mg). The ^{1}H and ^{13}C NMR data for these compounds agreed with reported data [7, 15–19]. The identity of cycloart-23-en-3β,25-diol and 25-methylenecycloartanol was confirmed by detailed 2D experiments.

Compound **1**. $C_{30}H_{48}O_2$, needles (from hexane) mp 196–198°, $[\alpha]_D$ + 10 ($CHCl_3$; *c* 0.10). IR: ν_{max}^{KB} cm^{-1}: 3560, 2950, 2850, 1720, 1650, 1640, 1450, 1370, 1360, 1260, 1180, 1140, 1110, 1040, 890, 880 and 850. ^{1}H and ^{13}C NMR: Table 1.

EIMS (rel. int.). 440 (71.7) $[M]^+$; 425 (30.2) $[M - Me]^+$, 367 (8.8) $[M - C_3H_5O_2]^+$, 359 (12.2), 218 (16.7), 205 (19.5), 204 (30.2), 203 (19.5), 191 (10.1), 190 (11.4), 189 (38.9), 178 (22.0), 177 (100), 176 (31.0), 175 (15.4), 163 (12.6), 161 (13.2), 149 (12.6), 157 (13.3), 135 (14.0), 133 (16.0), 123 (12.0), 121 (26.7), 119 (21.2), 109 (31.2), 105 (18.4), 95 (45.6), 93 (24.0), 91 (14.9), 83 (11.7), 81 (37.0), 79 (16.0) and 69 (34.4).

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REFERENCES

1. Bridson, D. L. (1975) *Kew. Bull.* **30**, 171.
2. Staugard, F. and Hedberg, I. (1989) *Traditional Medicinal Plants*. Ipelegeng Publishers, Gaborone, Botswana.
3. Aalbersberg, W. and Singh, Y. (1991) *Phytochemistry* **30**, 921.
4. Poehland, B. L., Cartel, B. K., Francis, T. A., Hyland, L. J., Allanundeen, W. and Troupe, N. (1987) *J. Nat. Prod.* **50**, 706.
5. Tai, K., Akahari, A. and Shingu, T. (1991) *Phytochemistry* **30**, 2796.
6. Budzikiewicz, H., Wilson, J. M. and Djerassi, C. (1963). *J. Am. Chem. Soc.* **85**, 3688.
7. Teresa, J. D. P., Urones, J. G., Marcos, I. S., Basabe, P., Cuadrado, M. J. and Moro, R. F. (1987) *Phytochemistry* **26**, 1767.
8. Ageta, H. and Arai, Y. (1983) *Phytochemistry* **22**, 1801.
9. Parsons, I. C., Gray, A. I., Lavaud, C., Massiot, G. and Waterman, P. G. (1991) *Phytochemistry* **30**, 1221.
10. Majumder, P. L., Maiti, R., Panda, S. K. and Mal, D. (1979) *J. Org. Chem.* **44**, 2811.
11. Tanaka, R., Ida, T., Takaoka, Y., Kita, S., Kamasako, W., Matsunaga, S. (1994) *Phytochemistry* **36**, 129.
12. Turnbull, J. H., Vasistha, S. K., Wilson, W. and Woodger, W. (1957) *J. Chem. Soc.* 569.
13. Whitham, G. H. (1959) *Proc. Chem. Soc.* London, 271.
14. Mangoni, L. and Belardini, M. (1963) *Tetrahedron Letters* 921.
15. Aplin, R. T., Arthur, H. R. and Hui, W. H. (1966) *J. Chem. Soc. (C)* 1251.
16. Majumder, P. L. and Pal, P. (1985) *Phytochemistry* **24**, 2120.
17. Ogunkoya, L. (1981) *Phytochemistry* **20**, 121.
18. Matsunaga, S., Tanaka, R. and Akagi, M. (1988) *Phytochemistry* **27**, 535.
19. Gaind, K. N., Singla, A. K., Boar, R. B. and Copsey, D. V. (1976) *Phytochemistry* **15**, 1999.

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ACRIDONE ALKALOIDS FROM *BOSISTOA TRANSVERSA*

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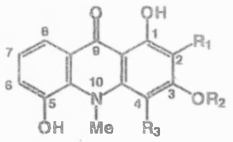
Key Word Index—*Bosistoa transversa*; Rutaceae; 2- and 4-prenylated 9-(10)-acridone alkaloids; bosistidine; bosistine; 4-(2ξ-hydroxy-3-methylbut-3-enyl)yukocritrine.

Abstract—Eight acridone alkaloids were isolated from *Bosistoa transversa*. Three of them are novel and have been identified as 1,3,5-trihydroxy-2-(2ξ-hydroxy-3-methylbut-3-enyl)-10-methylacridan-9-one (trivial name, bosistidine), 1,3,5-trihydroxy-2-(2ξ-hydroxy-3-methylbut-3-enyl)-4-(3-methylbut-2-enyl)-10-methylacridan-9-one (bosistine) and 1,3,5-trihydroxy-4-(2ξ-hydroxy-3-methylbut-3-enyl)-yukocritine. The structures were elucidated on the basis of NMR spectral data, notably NOESY and HMBC experiments.

INTRODUCTION

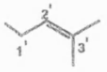
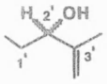
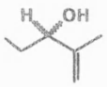
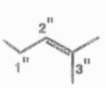
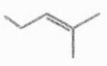
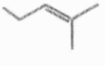
In continuation of our investigation into the chemistry of the genus *Bosistoa* [1, 2] we have undertaken a study of the aerial parts of *B. transversa* J. F. Bailey and C. T. White, a small- to medium-sized tree found in the rain forests of Queensland and northeast New South Wales [3]. In the present paper we report the isolation and identification of eight acridone alkaloids, of which three appear to be novel.



RESULTS AND DISCUSSION

By a combination of vacuum liquid chromatography (VLC), gel filtration and preparative TLC procedures, the hexane and ethyl acetate extracts of the leaf material afforded six acridones (**1**, **2** and **4**–**7**). Similar treatment of the stem bark again gave these alkaloids, plus the two additional acridones, **3** and **8**. The alkaloids were all yellow in colour and gave UV, IR and NMR spectra typical of the 1,3,5-oxygenated-*N*-methylacridan-9-one nucleus [4–6].

Five were known compounds which were identified, by direct comparison of their spectral data with that published, as citrusamine (**1**) [7], junosine (**2**) [8], *N*-methylataphylline (**5**) [9], yukocitrine (**7**) [10] and *N*-methylataphyllinine (**6**) [11]. The remaining compounds (**3**, **4** and **8**) were novel acridones, which had several NMR spectral features in common. There were strongly H-bonded hydroxyls at *ca* δ 15, three adjacent

	R_1	R_2	R_3
1	H	Me	H
2		H	H
3		H	H
4		H	
5		H	

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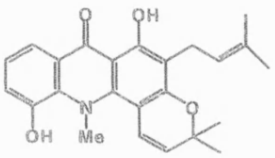

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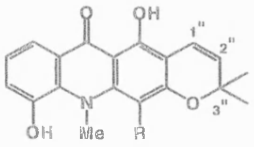
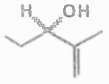


7 R = H

8 R = 

aromatic protons, one of which was deshielded and therefore placed *peri* to the carbonyl at H-8, an *N*-methyl group, and signals for a 2-hydroxy-3-methylbut-3-enyl moiety (Table 1).

In bosistidine (**3**), the only additional signal in the ^{1}H NMR spectrum was for a single A-ring proton, which must be placed at either C-2 or C-4. As this proton exhibited a strong NOE interaction with the *N*-methyl, it must be assigned to C-4 and bosistidine must be **3**.

Table 1. ^{1}H NMR (400 MHz, acetone-d_6) spectral data for compounds **3** and **8**

H	3	8
1-OH	15.26 *s*	14.70 *s*
4	6.45 *s*	—
5-OH	9.40 *s*	9.36 *s*
6	7.27 *dd* (7.8 1.4)	7.28 *dd* (7.9, 1.5)
7	7.12 *t* (7.8)	7.18 *t* (7.9)
8	7.90 *dd* (7.8, 1.6)	7.90 *dd* (7.9, 1.5)
N-Me	4.05 *s*	3.78 *s*
1′	3.15 *dd* (14.4, 8.0)	3.22 *dd* (14.4, 8.0)
	2.85 *dd* (14.4, 8.0)	3.18 *dd* (14.4, 8.0)
2′	4.40 *m*	4.64 *m*
2′-OH	5.35 *s*	5.35 *s*
H_2-4′	4.96 *s*	4.66 *s*
	4.76 *s*	4.54 *s*
3′-Me	1.85 *s*	1.76 *s*
1″	—	6.72 *d* (10.0)
2″	—	5.70 *d* (10.0)
$(Me)_2$	—	1.52 *s*
		1.50 *s*

The ^{1}H NMR spectrum of bosistine (**4**), showed in place of an A-ring proton, an additional 3-methyl-2-enyl group substituent. The problem of placing the two different prenyl substituents at C-2 and C-4 was resolved in a number of ways. (a) In the ^{13}C NMR spectrum (Table 2), the methylene of the 3-methylbut-2-enyl side-chain appeared at δ 27.3, which is characteristic of a C-4 substituent of this type [6]. (b) A ^{1}H–^{1}H NOESY experiment revealed a strong interaction between the *N*-methyl and the olefinic proton of the 3-methylbut-2-enyl side-chain at δ 5.40, again indicating that this side-chain is at C-4. (c) In an HMBC experiment (Table 2) long-range coupling (^3J) was observed between the methylene protons H-1′a and H-1′b of the 2-hydroxy-3-methylbut-3-enyl side-chain and the carbon nuclei resonating at δ 161.2 (C-1) and 164.5 (C-3), with a ^2J-coupling with C-2 (δ 108.3).

The third new alkaloid gave a ^{1}H NMR spectrum that showed signals for a 2,2-dimethyl chromene system and a 2-hydroxy-3-methylbut-3-enyl which must be located on ring A. The orientation of the pyran ring with respect to the acridone nucleus was suggested as linear by the UV spectrum [6, 10]. This required placement of the 2-hydroxy-3-methylbut-3-enyl side-chain at C-4; this was further confirmed by the observation of NOESY cross-peaks between the *N*-methyl and the exomethylene protons of the side-chain. The chemical shifts of the chromene system protons were very similar to those of yukocitrine (**7**) [10]. On the basis of these data, **8** has been assigned.

The three novel compounds all possessed the 2-hydroxy-3-methylbut-3-enyl side-chain, which has a chiral centre. Unfortunately, insufficient quantities were isolated to permit establishment of any absolute configurations.

Our study has revealed that *B. transversa* is a major source of 1,3,5-oxygenated-2,4-prenylated acridones. Many of the compounds isolated have previously been reported from two other rutaceous genera, *Atalantia* [9, 11] and *Citrus* [7, 8, 10]. Both of these genera are members of the sub-family Aurantioideae, while *Bosistoa* is part of the tribe Zanthoxyleae (sub-family Rutoideae). The co-occurrence of this unusual type of acridone in these taxonomically distance taxa of the Rutaceae is rather surprising. *Bosistoa transversa* appears to be chemically distinct from the previously studied *Bosistoa* species, *B. brassii* [2] and *B. floydii* [1], where the major metabolites are flavonoids. No flavonoids were detected in our study.

EXPERIMENTAL

Mps uncorr. UV: MeOH. IR: $CHCl_3$. EIMS: probe (90–130°) at 70 eV. FABMS: nitrobenzyl alcohol matrix. NMR: run in $CDCl_3$ or Me_2CO-d_6. NOESY and HMBC [12] expts were obtained on a Bruker AMX-400 instrument using standard Bruker microprograms.

Plant material. The sample investigated was collected on 22nd October 1991 near Mount French, Queensland. A voucher (T. G. Hartley 15168) is

Table 2. ^1H and ^{13}C NMR chemical shift data (400 MHz, acetone-d_6) and selected ^2J and ^3J H–C couplings for compound 4

Position	δ_H	δ_C	^2J	^3J
1	13.39 *s*	161.2		
2	—	108.4		
3	10.29 *s*	164.5		
4	—	110.1		
4a	—	150.5		
5a	—	139.1		
5	9.25 *s*	148.4		
6	7.27 *d* (8.0)	120.5	123.7, 148.4	117.3, 139.1
7	7.15 *t* (8.0)	123.7	117.3, 120.5	125.9, 148.4
8	7.77 *dd* (8.0, 1.2)	117.3	123.7, 125.9	120.5, 139.1, 183.5
8a	—	125.9		
9	—	183.5		
9a	—	105.0		
N-Me	3.69 *s*	48.5		139.1, 150.5
1′a	2.85 *dd* (14.4, 8.0)	30.0	77.5, 108.4	161.2, 164.5
1′b	3.19 *dd* (14.4, 8.0)	—		
2′	4.39 *dd* (8.0, 2.1)	77.5		
2′-OH	6.08 *br s*	—		
3′	—	148.2		
4′	4.82 *s*	110.5		
	5.04 *s*	—		
3′-Me	1.86 *br s*	18.7	148.2	77.5, 110.5
1″	3.56 *m*	27.3	110.0, 125.4	164.5
2″	5.40 *m*	125.4		
3″	—	131.5		
3″-Me	1.78 *s*	18.2	131.5	25.9, 125.4
3″-Me	1.69 *s*	25.9	131.5	18.2, 125.4

deposited at the Australian National Herbarium, Canberra.

Extraction and isolation of alkaloids. Ground leaves (400 g) and stem bark (80 g) were separately extracted (Soxhlet) with hexane, then EtOAc and finally MeOH. The hexane extract of the leaves (4 g) was fractionated by VLC over silica gel, eluting with petrol (bp 60–80°) containing increasing amounts of EtOAc. The 10–20% EtOAc eluate was passed through a column of Sephadex LH-20, eluting with $CHCl_3$, followed by prep. TLC (silica gel, $CHCl_3$–EtOAc, 9:1) to give **5** (10 mg) and **6** (5 mg). The EtOAc extract was passed through a column of Sephadex LH-20 followed by prep. TLC (silica gel, $CHCl_3$–EtOAc, 7:3) to give **1** (6 mg), **2** (4 mg), **4** (5 mg) and **7** (4 mg).

The hexane extract of the stem bark, when treated in an identical manner, gave two further alkaloids **3** (5 mg) and **8** (3 mg), together with **5** (20 mg) and **6** (7 mg). The EtOAc extract gave **1** (12 mg), **2** (12 mg), **4** (10 mg) and **7** (8 mg) on separation following the same procedures as used for the leaf extract.

Citrusamine (**1**). Yellow, amorphous. Found: $[M]^+$ 271.0841; $C_{15}H_{13}NO_4$ requires 271.0845. The spectral data (UV, IR, NMR, MS) were in agreement with that published [7].

Junosine (**2**). Orange needles from $CHCl_3$–MeOH, mp 220° (lit. [8] 210–213°). Found: $[M]^+$ 325.1325; $C_{19}H_{19}NO_4$ requires 325.1314. Spectral data (UV, IR, NMR, MS) were in agreement with that published [8].

1,3,5 - *Trihydroxy* - 2 - (2*ξ* - *hydroxy* - 3 - *methylbut* - 3 - *enyl*) - 10 - *methylacridan* - 9 - *one* (*bosistidine*) (**3**). Yellow, amorphous. $[\alpha]_D$ −15.1 (*c* 0.0018, $CHCl_3$). Found: $[M]^+$ 341.1263; $C_{19}H_{19}NO_5$ requires 341.1263. UV λ_{max} nm (log ε): 235 (4.20), 260 (4.27), 280 (4.35), 335 (3.90), 410 (3.50). IR γ_{max} cm^{-1}: 3400, 1630, 1560. ^1H NMR: Table 1. HREIMS: *m/z* (rel. int.): 341 (44), 323 (15), 308 (31), 270 (100), 236 (12).

1,3,5 - *Trihydroxy* - 2 - (2*ξ* - *hydroxy* - 3 - *methylbut* - 3 - *enyl*) - 4 - (3 - *methylbut* - 2 - *enyl*) - 10 - *methylacridan* - 9 - *one* (*bosistine*) (**4**). Yellow, amorphous. $[\alpha]_D$ −4.1 (*c* = 0.0013, $CHCl_3$). Found: $[M]^+$ 409.1879; $C_{24}H_{27}NO_5$ requires 409.1879. UV λ_{max} nm (log ε): 222 (4.16), 267 (4.36), 350 (4.15), 410 (3.55). IR γ_{max} cm^{-1}: 3580, 1710, 1625, 1573, 1455, 1285, 1040. ^1H and ^{13}C NMR: Table 2. HREIMS: *m/z* (rel. int.): 409 (12), 338 (85), 295 (5), 282 (100).

N-*Methylataphylline* (**5**). Orange needles from petrol–EtOAc, mp 187–190° (lit. [9] 190–193°). Found: $[M]^+$ 393.1926; $C_{24}H_{27}NO_4$ requires 393.1940. Spectral data (UV, IR, NMR, MS) were in agreement with that published [9].

N-*Methylataphyllinine* (**6**). Orange needles from petrol–EtOAc, mp 195° (lit. [11] 195–196°). Found: $[M]^+$ 391.1178; $C_{24}H_{25}NO_4$ requires 391.1793. Spectral data (UV, IR, NMR, MS) were in agreement with that published [11].

Yukocitrine (**7**). Yellow oil. Found: $[M]^+$ 323.1155; $C_{19}H_{17}NO_4$ requires 323.1158. Spectral data (UV, IR, NMR, MS) were in agreement with that published [10].

4-(2*ξ*-*Hydroxy*-3-*methylbut*-3-*enyl*)-*yukocitrine* (**8**).

Yellow oil. $[\alpha]_D$ +16.4 (*c* 0.0005, $CHCl_3$). Found: FABMS $[M + 1]^+$ 408 = $C_{24}H_{25}NO_5$. UV λ_{max} nm (log ε): 225 (4.15), 266 (4.30), 300 (4.65), 305 (34.15), 335 (3.55), 405 (3.55). IR γ_{max} cm^{-1}: 3320, 3010, 1650, 1608, 1555. ^1H NMR: Table 1. FABMS: *m/z* (rel. int.): 408 $[M + 1]^+$, 336 (100), 165 (21).

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REFERENCES

1. Auzi, A. A., Gray, A. I., Hartley, T. G., Waigh, R. D. and Waterman, P. G. (1994) *Phytochemistry* **36**, 535.
2. Parsons, I. C., Gray, A. I. and Waterman, P. G. (1992) *J. Nat. Prod.* **56**, 46.
3. Hartley, T. G. (1977) *J. Arnold Arbor.* **58**, 426.
4. Groger, D. (1988) *Pharmazie* **43**, 815.
5. Diment, J. A., Ritchie, E. and Taylor, W. C. (1967) *Aust. J. Chem.* **20**, 1723.
6. Furukawa, H., Yogo, M. and Wu, T. S. (1983) *Chem. Pharm. Bull. (Tokyo)* **31**, 3084.
7. Ju-ichi, M., Inoue, M., Ito, C. M., Furukawa, H. and Kajiura, I. (1987) *Heterocycles* **26**, 1873.
8. Ju-ichi, M., Inoue, M., Ito, C. M. and Aoki, K. (1986) *Heterocycles* **24**, 1595.
9. Govindachari, T. R. and Viswanathan, N. (1970) *Tetrahedron* **26**, 2905.
10. Takemura, Y., Uchida, H., Ju-ichi, M., Ito, C., Nakagawa, K., Ono, T. and Furukawa, H. (1992) *Heterocycles* **34**, 2123.
11. Shah, J. S. and Sabata, B. K. (1982) *Indian J. Chem.* **21**, 16.
12. Bax, A. and Summers, M. F. (1986) *J. Am. Chem. Soc.* **108**, 2093.

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PHLOROGLUCINOL DERIVATIVES FROM LEAVES OF *BOSISTOA PENTACOCCA*

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Key Word Index—*Bosistoa pentacocca*; *B. pentacocca* var. *connaricarpa*; *B. pentacocca* var. *dryanderensis*; Rutaceae; leaves; phloroglucinol derivatives; pentacoccols.

Abstract—Leaves from four collections of *Bosistoa pentacocca*, two of the variety *connaricarpa* and one each of the type variety and var. *dryanderensis*, have been examined. In each, the major metabolite isolated was characterized as 2-(3,7-dimethyloct-2,5-dienyl)-6-(3-methylbut-2-enyl)phloroglucinol-1-acetate (trivial name pentacoccol). Four other pentacoccol derivatives were obtained as minor components, pentacoccol-5-methyl ether (from var. *pentacocca* and var. *dryanderensis*), 2,2-dimethyl-3ζ,7-dihydroxy-5-acetoxy-6-(3,7-dimethyloct-2,5-dienyl)-3,4-dihydrobenzo-[2*H*]-pyran (3′-hydroxydihydropyranopentacoccol from var. *connaricarpa*), 2-(1-hydroxy-1-methylethyl)-4-acetoxy-5-(3,7-dimethyloct-2,5-dienyl)-6-hydroxybenzo-[2*H*]-furan (2′-(1-hydroxyosopropyl)furanopentacoccol from var. *pentacocca*) and 4-acetoxy-5-(3,7-dimethyloct-2,5-dienyl)-6-hydroxybenzo-[2*H*]-furan (furanopentacoccol from var. *dryanderensis*). All compounds were identified on the basis of their spectroscopic data.

INTRODUCTION

The genus *Bosistoa* F. Muell. ex Benth., as currently delineated by Hartley [1], consists of seven species of small trees found in the rain forests of New South Wales and Queensland. In a series of studies, we have reported on flavonoids from *B. brassii* Hartley [2] and *B. medicinalis* (F. M. Bailey) Hartley [3], a xanthine alkaloid from *B. floydii* Hartley [4] and acridone alkaloids from *B. transversa* [5] and *B. selwynii* Hartley [6].

In the present work, we wish to report the results of a study on the leaves of *B. pentacocca* (F. Muell.) Baillon. Hartley [1] recognized three varieties of this species: var. *pentacocca* occurring in south-east Queensland and north-east New South Wales, var. *connaricarpa* (Domin) Hartley occurring in east central Queensland from Bowen south to Gin-Gin, and var. *dryanderensis* Hartley also occurring in east central Queensland around Mount Dryander and MacKay. A previous investigation on *B. pentacocca*, reported under the illegitimate name *B. sapindiformis*, yielded the pentacyclic triterpenes, taraxerol and taraxerol methyl ether [7]. The only other phytochemical report citing the genus was for *B. cuodiiformis*, which yielded acetophenones, furoquinoline alkaloids, a limonoid and the triterpene, bosistoin [8]. *Bosistoa euodiiformis* is now considered [1] to be a species of *Acradenia* rather than *Bosistoa*.

RESULTS AND DISCUSSION

The major compound isolated from each of the four samples gave an ion at *m/z* 395 $[M+Na]^+$ in FAB mass spectra, which corresponded with an empirical formula, $C_{23}H_{32}O_4$. Two major fragments in the HREI mass spectra indicated the loss of C_4H_7 and C_9H_{15} fragments, which suggested the presence of C-prenyl and C-geranyl units, while a major ion at *m/z* 43 was typical of an acetoxyl substituent.

The ^{1}H NMR spectra (Table 1) confirmed the presence of an acetyl methyl and of 3-methylbut-2-enyl and geranyl moieties. This accounted for all but three protons, which were observed as an aromatic proton singlet at δ 6.36 and two aromatic phenols at δ 8.08 (*br*). The ^{13}C NMR spectrum (Table 1) revealed signals typical of prenyl and geranyl side-chains and the acetoxy, leaving six further carbons, the single aromatic methine (δ 101.3), two equivalent shielded quaternary carbons (δ 113.1) and three deshielded oxygenated aromatics (δ 150.4 and 154.7—double inten-

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Table 1. ^{1}H and ^{13}C NMR chemical shift values for compounds **1** and **2**

	^{1}H		^{13}C	
C/H	**1**	**2**	**1**	**2**
1			150.4	148.2
2			113.1	111.7*
3 (OH)	8.08 *s*		154.7	156.9
4	6.38 *s*	6.30 *s*	101.3	98.1
5 (OH)	8.08 *s*		154.7	154.1
6			113.1	114.9*
1-OCOMe	2.25 *s*	2.29 *s*	169.3, 20.8	169.5, 20.7
5-OMe		3.74 *s*		55.8
1′	3.08 *d* (6.6)	3.17 *d* (6.7)	23.8*	23.8
2′	5.12 *t* (6.6)	5.20 *t* (6.7)	124.3	122.0
3′			130.7	134.4
3′-Me	1.71 *s*	1.77 *s*	25.9	25.8
3′-Me	1.63 *s*	1.72*s*	17.8	17.9
1″	3.08 *d* (6.6)	3.15 *d* (6.8)	23.9*	23.2
2″	5.12 *t* (6.6)	5.08 *t* (6.8)	124.3	122.8
3″			134.5	134.8
3″-Me	1.72 *s*	1.72 *s*	16.3	16.2
4″	1.94 *m*	1.95 *m*	40.3	39.8
5″	2.08 *m*	2.06 *m*	27.4	26.8
6″	5.12 *t* (6.6)	5.08 *t* (6.7)	125.3	124.5
7″			131.7	131.3
7″-Me	1.63 *s*	1.63 *s*	25.9	25.8
	1.57 *s*	1.57 *s*	17.8	17.8

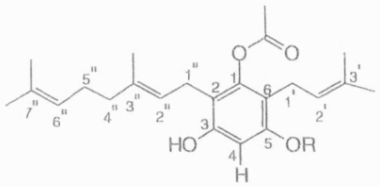
1 in acetone-d_6, **2** in $CDCl_3$.
* Signals interchangeable.

sity). This required the presence of a systematically trioxygenated ring system (e.g. phloroglucinol), in which two of the non-oxygenated carbons were substituted by the 3-methylbut-2-enyl and geranyl groups; one of the oxygenated positions was acetylated.

The positions of the various substituents were established by means of long-range heteronuclear coupling (HMBC) experiments [9] on the basis of the following observations. The single aromatic proton revealed two interactions, with the two equivalent resonances at δ 154.7 (^2J) and with the second equivalent pair at δ 113.1 (^3J). From the two phenolic hydroxyl protons, ^2J interactions were revealed to the carbons resonating at δ 154.7, while there was an intense ^3J interaction to the signals at δ 101.3 and 113.1. These observations required the compound to have the symmetrical structure **1**. Unequivocal assignment of the carbon resonances (Table 1) was confirmed from the full analysis of HMBC and HC-COBI [10] experiments. Compound **1** appears to be novel and has been assigned the trivial name, pentacoccol.

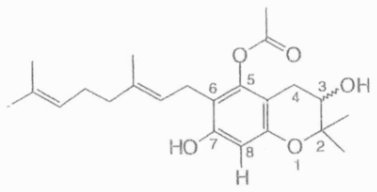
A very similar compound differing only in the methylation of one of the aromatic hydroxyls ($C_{24}H_{34}O_4$ from HREI mass spectrum, δ 3.74 (3H), 55.7) was obtained from *B. pentacocca* var. *pentacocca* and *B. pentacocca* var. *dryanderensis*. The ^{13}C NMR spectrum (Table 1) indicated the phloroglucinol oxygenation pattern and, together with the ^{1}H NMR spectrum, that the same prenyl and geranyl side-chains were present. The relatively shielded resonance for the methyoxyl carbon at *ca* δ 56 (cf. 60–62 ppm where both *ortho*-positions are substituted) required placement adjacent to the aromatic proton [11]. Assignment to C-5, adjacent to the prenyl substituent, was resolved from a HMBC experiment, where both H-1′ of the prenyl side-chain and the OMe protons showed a ^3J coupling to the carbon at δ 156.9. On this basis, the compound could be characterized as pentacoccol-5-methyl ether (**2**), which is again novel.

Three further phloroglucinol derivatives were obtained as minor components, each based on cyclization of the prenyl side-chain with the adjacent hydroxyl. Both samples of *B. pentacocca* var. *connaricarpa* yielded a compound which analysed (HREI mass spectrum) for $C_{23}H_{32}O_5$. The NMR spectra (Table 2) revealed the presence of the phloroglucinol system with the acetoxyl and geranyl substituents but the signals for the prenyl unit were not present, being replaced, in the ^{1}H NMR spectrum, by a CH_2-CH(O)- spin system and two methyls and, in the ^{13}C NMR spectrum, by a methylene, an oxymethine, an oxygenated quaternary sp^3 carbon and two methyls. These resonances could be assigned to a 3-hydroxy-2,2-dimethyldihydropyran system which could be formed by cyclization of a 2,3-dihydroxy-3-methylbutanyl side-chain and the 5-OH with the loss of the elements

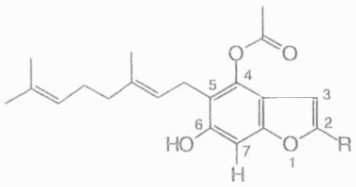


1 R = H

2 R = Me



3



4 R = $C(OH)Me_2$

5 R = H

of water. This was confirmed by an HMBC experiment (Table 2). Thus, this compound is identified as 5-acetoxy-3,4-dihydro-3ξ,7-dihydroxy-6-(3,7-dimethyloct-2,5-dienyl)-2,2-dimethyl-[2H]-benzopyran (**3**), to which we assign the trivial name, 3′ξ-hydroxy-dihydropyranopentacoccol.

A minor isolate from *B. pentacocca* var. *pentacocca* gave a $[M]^+$ for $C_{23}H_{30}O_5$ (HREI mass spectrum). In this case, the signals originating from the modified prenyl group were an aryl methine at δ 6.87 showing a small coupling (0.8 Hz) to the phloroglucinol proton (cf. H-4 in **1**) and geminal methyls resonating at δ 1.64. The ^{13}C NMR spectrum and a HC-COBI experiment revealed that the methine carbon resonated at δ 95.7, the methyls at δ 28.8. An HMBC experiment showed that the methine and methyl protons coupled with quaternary carbons at δ 69.4 and 162.0, which must be oxygen-bonded sp^3 and sp^2 carbons, respectively. There are two plausible structures that could fit these data, a 3-hydroxy-2,2-dimethylpyran or a 2-(1-hydroxyisopropyl)furan. The resonance positions observed were very similar to those noted previously for the acetophenone, furostipitol [12]. In particular, the resonance for the sp^3 quaternary carbon (δ 69.4) was typical of the 1-hydroxyisopropyl group, whereas, in the comparable C-2′ position of benzopyrans, this occurs at *ca* δ 78 (cf. **3**). On this basis, the compound is identified as 2-(1-hydroxy-1-methylethyl)-4-acetoxy-6-hydroxybenzofuran, to which we have assigned the trivial name 2′-(1-hydroxyisopropyl) furanopentacoccol (**4**).

From *B. pentacocca* var. *dryanderensis* another minor compound was obtained which analysed (HREI mass spectrum) for $C_{20}H_{24}O_4$. The ^1H NMR spectrum once again identified the phloroglucinol ring, and geranyl and acetoxyl substituents. The remaining two methines formed a simple spin system (J = 2.2 Hz) typical of a furan ring. Insufficient material was available to obtain a satisfactory ^{13}C NMR spectrum but this compound must be a simple derivative furanopentacoccol (**5**).

A search of the Dictionary of Natural Products [13] failed to show any compounds directly analogous to the pentacoccols. Within the Rutaceae, a number of acetophenones with a phloroglucinol oxygenation pattern substituted with prenyl, geranyl and farnesyl substituents are known, many of which exhibit similarly modified and cyclized side-chains [14]. In *B. pentacocca*, it seems that the phloroglucinol system has been biosynthesized from the combination of three acetate units, whereas, in the acetophenones, four acetate units are involved. The differences in pro-

Table 2. ^1H and ^{13}C NMR chemical shift values for compound 3

	^1H	^{13}C	^2J	^3J
2		77.1		
2-Me	1.34 *s*	25.9	77.1	25.9, 69.5, 152.3
	1.29 *s*	25.9	77.1	25.9, 69.5, 152.3
3	3.75 *t* (5.2)	69.5		105.5
4	2.50 *br d*	26.8		
	2.70 *br d*			
4a		105.5		
5		148.5		
6		112.9		
7		154.8		
8	6.25 *s*	102.9	152.3, 154.8	105.5, 112.9
8a		152.3		
OCOMe	2.31 *s*	169.0, 20.7		
1″	3.17 *d* (6.7)	23.6	112.9, 121.7	138.5, 148.5, 154.8
2″	5.17 *t* (6.7)	121.7	23.6	16.3, 39.9
3″		138.5		
3″-Me	1.81 *s*	16.3	138.5	39.9, 121.7
4″	2.03 *m*	39.9	26.6, 138.5	16.3, 121.7
5″	2.08 *m*	26.6	39.9, 124.0	132.2
6″	5.05 *t* (6.8)	124.0		
7″		132.2		
7″-Me	1.67 *s*	25.9	132.2	17.9, 124.0
	1.59 *s*	17.9	132.2	25.9, 124.0

Spectra in $CDCl_3$.

duction of minor components are not considered to have taxonomic significance; rather, the codominance of pentacoccol is considered to support the close affinity between these taxa proposed by Hartley [1].

EXPERIMENTAL

Extractions were carried out using a Soxhlet apparatus using, sequentially, petrol (bp 60–80°), EtOAc and MeOH as solvents. UV: MeOH. IR: solvent-free film. HREIMS: 70 eV, FABMS: NOBA-matrix. NMR spectra were obtained on a Bruker AMX-400 instrument using standard Bruker microprograms for 2D expts [15].

Plant material. Bosistoa pentacocca var. *pentacocca*: voucher: T. G. Hartley 15170 collected 22 October 1991 on Mount French, Moreton Bay District, Queensland, *B. pentacocca* var. *connaricarpa*: voucher: T. G. Hartley 13201 collected 11 April 1993, State of Forest 50, Glenbar, Wide Bay District, Queensland, voucher: P. Vorster 9398 collected 18 January 1992, Mount Colosseum National Park, Port Curtis District, Queensland, and *B. pentacocca* var. *dryanderensis*: voucher P. Vorster 9411 collected 19 January 1992, Mount Dryander, North Kennedy District, Queensland.

Extraction of B. pentacocca *var.* pentacocca. Dried, milled leaves (230 g) yielded a concentrate (2.5 g) from the petrol extract. CC of this material over Sephadex LH-20, eluting with $CHCl_3$ removed the chlorophylls and yielded a fr. containing **1** as an amorphous solid (1 g). The EtOAc concentrate (5 g) was treated in a similar manner, eluting the Sephadex LH-20 with 10% MeOH in $CHCl_3$ which yielded, in sequence of elution, sitosterol (30 mg), **2** and **5**. Prep. TLC of **2** and **5** (silica gel; petrol–EtOAc, 9:1) yielded pure **2** (100 mg, R_f 0.67) and **4** (4 mg, R_f 0.14).

Extraction of B. pentacocca *var.* connaricarpa (*Hartley* 13201). Dried, milled leaves (290 g) yielded a petrol concentrate (6 g) and an EtOAc concentrate (10 g). These were subjected to identical treatment to *B. pentacocca* var. *pentacocca* to yield, from the petrol extract, **1** (425 mg), and from the EtOAc extract, sitosterol (20 mg) and **3** (3.5 mg, R_f 0.45, petrol–EtOAc, 1:1).

Extraction of B. pentacocca *var.* connaricarpa (*Vorster* 9398). Dried, milled leaves (185 g) yielded a petrol concentrate (2 g) and an EtOAc concentrate (5 g). These were subjected to identical treatment noted above, to yield, from the petrol extract, **1** (1.2 g), and from the EtOAc extract, sitosterol (30 mg) and **3** (5 mg).

Extraction of B. pentacocca *var.* dryanderensis. Dried, milled leaves (400 g) yielded a petrol concentrate (15 g) and an EtOAc concentrate (10 g). These were subjected to identical treatment to that noted above, to yield, from the petrol extract, **1** (1.5 g), and from the EtOAc extract, sitosterol (30 mg), **2** (100 mg) and **5** (4 mg, R_f 0.74 in petrol–EtOAc, 9:1).

Pentacoccol (**1**). Amorphous solid. FABMS: *m/z*

Table 3. ^{1}H and ^{13}C NMR chemical shift data and long-range H-C coupling for compounds **4**, and ^{1}H NMR data for **5**

H	^{1}H	^{13}C	^2J	^3J	**5**
2		162.0			7.47 *d* (2.2)
3	6.87 *d* (0.8)	95.9**	115.9, 162.0	141.1, 154.4	6.52 *br d* (2.2)
3a		115.9			
4		141.1			
5		115.7*			
6		153.3			
7	6.32 *d* (0.8)	95.7**	153.3, 154.4	115.7, 115.9	6.96 *br s*
7a		154.4			
OCOMe	2.42 *s*	169.1, 20.9		169.1	2.40 *s*
1″		69.4			
1″-Me	1.64 *s*	28.8	69.4	28.8, 162.0	
	1.64 *s*	28.8	69.4	28.8, 162.0	
1′	3.30 *d* (6.8)	23.7	115.7, 121.5	138.7, 141.1, 153.3	3.36 *d* (6.6)
2″	5.19 *t* (6.8)	121.5			5.21 *t* (6.6)
3″		138.7			
3″-Me	1.83 *s*	16.4	138.7	39.8, 121.5	1.80 *s*
4″	2.10 *m*	39.8	26.6, 138.7	16.4, 123.9	2.06 *m*
5″	2.16 *m*	26.6	39.8, 123.9	132.2	2.12 *m*
6″	5.03 *t* (6.6)	123.9			5.05 *t* (7.0)
7″		132.2			
7″-Me	1.72 *s*	25.8	132.2	17.9, 123.9	1.68 *s*
	1.59 *s*	17.9	132.2	25.8, 123.9	1.60 *s*

Spectra in $CDCl_3$.

*/** Signals with same number of * are interchangeable.

395 $[M+Na]^+$. UV λ_{max} nm (log ε): 208 (4.18), 225 (3.99), 273 (3.63). IR ν_{max} cm^{-1}: 3436, 2924, 1744, 1633, 1598, 1466, 1370, 1199, 1050. NMR: Table 1. EIMS *m/z* (rel. int.): 249 (35), 207 (70), 151 (52), 123 (100).

Pentacoccol 5-methyl ether (**2**). Oil. FABMS: *m/z* 409 $[M+Na]^+$. HREIMS: found $[M]^+$ *m/z* 386.2485; $C_{24}H_{34}O_4$ requires 386.2458. UV λ_{max} nm (log ε): 210 (4.28), 226 (4.05), 274 (3.70). IR ν_{max} cm^{-1}: 3436, 2916, 1735, 1620, 1487, 1433, 1368, 1208, 1164, 1001. NMR: Table 1. EIMS *m/z* (rel. int.): 363 (33), 247 (12), 221 (48), 219 (100), 166 (8), 123 (43).

3′-Hydroxydihydropyranopentacoccol (**3**). Oil. $[\alpha]_D$ −7.5° (*c* 0.002, $CHCl_3$). HREIMS: found $[M]^+$ *m/z* 388.2196; $C_{23}H_{32}O_5$ requires 388.2250. UV λ_{max} nm (log ε): 209 (4.47), 225 (4.17), 285 (3.87). IR ν_{max} cm^{-1}: 3393, 2921, 1738, 1627, 1599, 1447, 1369, 1201, 1117, 1063. NMR: Table 2. EIMS *m/z* (rel. int.): 388 (56), 265 (63), 223 (100).

2′-(1-Hydroxyisopropyl)furanopentacoccol (**4**). Oil. HREIMS: found $[M]^+$ *m/z* 386.2057; $C_{23}H_{30}O_5$ requires 386.2094. UV λ_{max} nm (log ε): 210 (4.32), 249 (3.95), 295 (3.81). IR ν_{max} cm^{-1}: 3360, 2924, 1742, 1633, 1467, 1369, 1208, 1163, 1105, 1074. NMR: Table 3. EIMS *m/z* (rel. int.): 386 (40), 344 (39), 327 (27), 221 (100), 123 (31).

Furanopentacoccol (**5**). Oil. HREIMS: found $[M]^+$ *m/z* 328.1665; $C_{20}H_{24}O_4$ requires 328.1675. UV λ_{max} nm (log ε): 213 (4.47), 228 (4.10), 273 (3.93). IR ν_{max} cm^{-1}: 3452, 2919, 1735, 1623, 1469, 1445, 1370, 1164, 1049, 1043. NMR: Table 3. EIMS *m/z* (rel. int.): 328 (86), 286 (84), 163 (100).

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REFERENCES

1. Hartley, T. G., *Journal of the Arnold Arboretum*, 1977, **58**, 416.
2. Parsons, I. C., Gray, A. I., Waterman, P. G. and Hartley, T. G., *Journal of Natural Products*, 1993, **56**, 46.
3. Auzi, A. A., Hartley, T. G., Waigh, R. D. and Waterman, P. G., *Flavonoids and Bioflavonoids: Proceedings of the International Bioflavonoid Symposium, Vienna*, ed. S. Antus, M. Gabor and K. Vetschera. Akademiai Kiado, Budapest, 1996, p. 383.
4. Auzi, A. A., Gray, A. I., Hartley, T. G., Waigh, R. D. and Waterman, P. G., *Phytochemistry*, 1994, **36**, 535.

5. Auzi, A. A., Hartley, T. G., Waigh, R. D. and Waterman, P. G., *Phytochemistry*, 1996, **42**, 235.
6. Auzi, A. A., Waigh, R. D. and Waterman, P. G., *Journal of Pharmacy and Pharmacology*, 1994, **46**, (Suppl.) 1086.
7. Croft, J. A., Ritchie, E. and Taylor, W. C., *Australian Journal of Chemistry*, 1975, **28**, 2093.
8. Croft, J. A., Ritchie, E. and Taylor, W. C., *Australian Journal of Chemistry*, 1976, **29**, 695.
9. Bax, A. and Summers, M. F., *Journal of the American Chemical Society*, 1986, **108**, 2093.
10. Bax, A., *Journal of Magnetic Resonance*, 1983, **53**, 517.
11. Panichpol, K. and Waterman, P. G., *Phytochemistry*, 1978, **17**, 1363.
12. Parsons, I. C., Gray, A. I., Hartley, T. G. and Waterman, P. G., *Phytochemistry*, 1994, **37**, 565.
13. *The Dictionary of Natural Products on CD-ROM*, Version 5.2. Chapman and Hall, London, 1996.
14. Quader, M. A., Armstrong, J. A., Gray, A. I., Hartley, T. G. and Waterman, P. G., *Biochemical Systematics and Ecology*, 1991, **19**, 171.
15. Quader, M. A., Gray, A. I., Waterman, P. G., Lavaud, C., Massiot, G. and Sadler, I. H., *Tetrahedron*, 1991, **47**, 3611.

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BISABOLENE SESQUITERPENES AND FLAVONOIDS FROM *FRIESODIELSIA ENGHIANA*

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Key Word Index—*Friesodielsia enghiana*; Annonaceae; bisabolene sesquiterpenes; flavonoids; 2-hydroxyflavonoids; flavones; flavanones.

Abstract—An investigation of the stem bark of *Friesodielsia enghiana* led to the isolation of benzyl benzoate and benzyl 2-hydroxybenzoate, two bisabolene sesquiterpenes and nine flavonoids, two of which were new. The new compounds were identified by analysis of their spectroscopic data as 2,5-dihydroxy-7-methoxy-8-methylflavanone and 2,5-dihydroxy-7-methoxy-6-methylflavanone. Three of the co-occurring flavones also have 6- and/or 8-C-methylation. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Friesodielsia van Steenis is a genus of Annonaceae with about 60 species found in Africa and Asia [1]. Until 1948, species belonging to this genus were placed under *Oxymitra* [2]. The first phytochemical report on the genus was made in 1960, in which the presence of alkaloids in an unidentified species was noted [3]. Since then only two species have been investigated, *F. kingii* (syn. *Oxymitra kingii*) from which hexahydroxanthenic derivatives and a flavanone were isolated [4], and *F. velutina* (syn. *O. velutina*) which yielded flavonoids, phenylpropanoids, sterols and alkaloids [5]. In this report we present the results of a phytochemical investigation on the stem bark of *F. enghiana* (Diels) Verdc., a large woody climber found in the tropical rain forest of West Africa, from Sierra Leone to Zaire [1].

RESULTS AND DISCUSSION

The stem bark of *F. enghiana* was successively extracted with petrol (bp 60–80°) and $CHCl_3$. The concentrated petrol extract, after repeated column chromatography and preparative TLC on silica gel, yielded benzyl benzoate (**1**), benzyl 2-hydroxybenzoate (**2**) [6], two bisabolene sequiterpenes, β-bisabodol (**3**) [7] and gossonorol (**4**) [8], and the flavonoids 5-hydroxy-7-methoxy-8-methylflavone (**5**) [10], 5-hydroxy-7-methoxy-6-methylflavone (**6**) [10], 5-hydroxy-7-methoxyflavone (**7**) [11], 5-hydroxy-7-methoxy-6,8-dimethylflavone (**8**) [10], 5-hydroxy-7-methoxyflavanone (**9**) [12], 2,5-dihydroxy-7-methoxy-8-methylflavanone (**10**) and 2,5-dihydroxy-7-methoxy-6-methylflavanone (**11**). The $CHCl_3$ extract after similar treatment gave compounds **1**, **5**, **6**, **9**, **10** and **11**, and in addition 2,5-dihydroxy-7-methoxyflavanone (**12**) [13, 14] and 5,7-dihydroxy-6-methylflavanone (**13**) [15]. Compounds **1**, **5** and **6** formed the major constituents of the petrol extract; **10** and **11** the major constituents of the $CHCl_3$ extract. All the known compounds were identified by comparison of their spectroscopic data with those published.

Compounds **10** and **11** were isolated as a mixture (**10**:**11** = 1:0.94) as shown by the ^{1}H NMR. The HR-EIMS gave a single molecular ion peak at m/z 300.1006 which analysed for $C_{17}H_{16}O_5$. The UV analysis showed a strong absorption at 276 nm and a shoulder at 336 nm, which is typical of flavanones [10], and the IR spectrum indicated hydroxyl and carbonyl functions. The ^{1}H NMR spectrum of **10**, the major compound of the mixture, showed a singlet at δ 12.64, and aromatic multiplets at δ 7.43 (3H) and δ 7.68 (2H), suggesting a 5-hydroxyflavanone with an unsubstituted phenyl moiety (B-ring) which was supported by the fragment at m/z 77 in the mass spectrum. The ^{1}H NMR further showed an aromatic methyl singlet (8-Me), an aromatic proton singlet (H-6) and a methoxyl singlet (7-MeO). The positions of H-6 and 8-Me were unambiguously assigned from an HMBC experiment (Table 1) which showed ^3J couplings of the former with C-8 and C-10, and the latter with C-7 and C-9.

The absence of a double doublet in the region of δ 5.20 suggested the absence of an H-2 proton which normally couples with the H-3 protons in flavanones [11]. That this proton was substituted by an OH was supported by the presence of a doubly oxygenated sp^3 carbon signal at δ 101.8 (C-2) in the ^{13}C NMR

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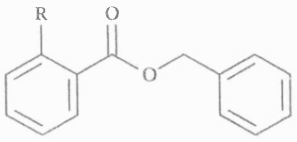
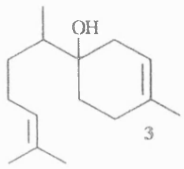


	R
1	H
2	OH



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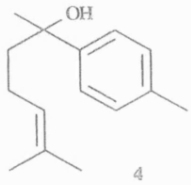

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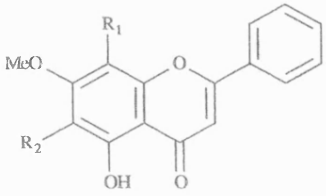
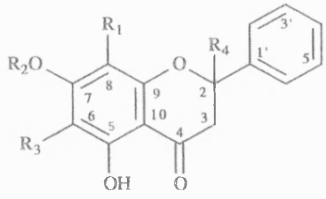


	R_1	R_2
5	Me	H
6	H	Me
7	H	H
8	Me	Me



	R_1	R_2	R_3	R_4
9	H	Me	H	H
10	Me	Me	H	OH
11	H	Me	Me	OH
12	H	Me	H	OH
13	H	H	Me	H

Table 1. Long-range ^{1}H–^{13}C correlations shown by **10** in HMBC experiment

H	^{3}J	^{2}J
H-3	101.8 (C-2), 195.9 (C-4)	
H-6	162.2 (C-5), 166.3 (C-7)	106.0 (C-8), 102.5 (C-10)
8-Me	106.0 (C-8)	166.3 (C-7), 155.7 (C-9)
H-2′/6′	142.4 (C-1′), 128.9 (C-3′)	101.8 (C-2), 129.4 (C-4′)

spectrum. The position of this carbon was established from ^{3}J coupling with the H-2′/6′ protons in the HMBC experiment. This 2-OH gave a doublet at δ 2.70 while the H-3 protons formed an *AB* system with resonance signals at δ 2.55 (H-3_{ax}) and δ 2.83 (H-3_{eq}). These data led to the assignment of the structure of **10** as 2,5-dihydroxy-7-methoxy-8-methylflavanone. The small J of 2.5 Hz for the 2-OH proton must be due to long range W-coupling with the 3-H_{ax} (Fig. 1) thus suggesting a pseudo-equatorial configuration of the phenyl substituent.

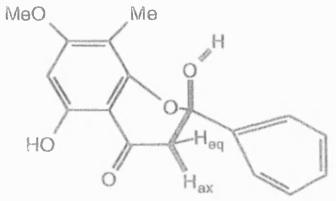


Fig. 1. W-coupling between H_{ax} and 2-OH.

Compound **11** gave signals similar to and sometimes overlapping with those of **10** in both the ^{1}H and ^{13}C NMR spectra (Table 2). Analysing the HC-COB1 and HMBC experiments the structure of this compound was established as the corresponding 2,5-dihydroxy-7-methoxy-6-methylflavanone.

Although flavonoids are widespread in nature, the occurrence of such stable hemiacetals with hydroxyl substitution at position C-2, as in compounds **10**, **11** and **12**, is rare. Previously, only 7-methoxy-2,5-dihydroxyflavanone from *Populus nigra* [14] and *Uvaria rufas* [13], 7-glucosyloxy-2,5-dihydroxyflavanone from *Mallus* sp. [16] and 6-formyl-2,5,7-trihydroxy-8-methylflavanone and 8-formyl-2,5,7-trihydroxy-6-methylflavanone from *Unona lawii* [17], have been encountered. The biogenesis of these compounds remains unknown. However, they have been regarded as precursors of 5-hydroxyflavones because of the ease with which they are dehydrated to the latter [17]. In this regard, the co-occurrence of the 2,5-dihydroxyflavanones and their corresponding 5-hydroxyflavones in the stem bark of *F. enghiana* is noteworthy.

EXPERIMENTAL

Mps: uncorr.; UV-MeOH; IR-$CHCl_3$; NMR experiments were run on a Bruker AMX 400 instrument; HR-EIMS were obtained on a AEI MS 902 double

Table 2. ^{1}H and ^{13}C NMR data for **10** and **11**

	δ ^{1}H*			$\delta^{13}C$†	
Position	**10**	**11**	**12**	**10**	**11**
2				101.8 *s*	101.3 *s*
3_{eq}	2.83 *d* (*J* = 17 Hz)	2.81 *d* (*J* = 17 Hz)	2.77 *d* (*J* = 17 Hz)	48.4 *t*	48.7 *t*
3_{ax}	2.55 *dd* (*J* = 2.6, 17 Hz)	2.60 *dd* (*J* = 2.4, 17 Hz)	2.56 *dd* (*J* = 2.6, 17 Hz)		
4				195.0 *s*	194.6 *s*
5				162.2 *s*	160.3 *s*
6	6.02 *s*		6.13 *d* (*J* = 2.0 Hz)	92.8 *d*	106.7 *s*
7				166.3 s	165.9 s
8		5.99 *s*	6.19 *d* (*J* = 2.0 Hz)	106.0 *s*	91.9 *d*
9				155.7 *s*	158.0 *s*
10				102.5 *s*	102.5 *s*
1′				142.4 *s*	142.1 *m*
2′/6′	7.43 *m*	7.39 *m*		125.2 *d*	125.2 *d*
3′/5′	7.14 *m*	7.09 *m*		128.9 *d*	128.9 *d*
4′	7.14 *m*	7.09 *m*		129.4 *d*	129.4 *d*
6-Me		2.22 s			7.1 *q*
7-OMe	3.16 *s*	3.25 *s*	3.13 *s*	56.1 *q*	56.0 *q*
8-Me	2.28 *s*			8.0 *q*	
2-OH	2.70 *d* (*J* = 2.7 Hz)	3.02 *d* (*J* = 2.6 Hz)	2.84 *d* (*J* = 2.6 Hz)		
5-OH	12.64 *s*	12.57 *s*	12.56 *s*		

*Solution in C_6D_6 referenced to C_6D_6 at δ 7.16 (400 MHz).
†Solution in $CDCl_3$ referenced to $CHCl_3$ at δ 77.23 (100 MHz).

focusing spectrometer using direct probe insertion at 120° and 70 eV.

Plant material. The stem bark of *F. enghiana* was collected from the Bobre Forest Reserve, Ghana, in August 1993, and was identified by comparison with herbarium specimens at the Forest Herbarium of the forestry Department, Kumasi.

Extraction and isolation of compounds. Oven-dried (40°) powdered stem bark (500 g) was Soxhlet extracted successively with petrol (bp 60–80°) and $CHCl_3$. The extracts were evaporated at 30° under red. pres. to give 11.08 and 10.51 g extract, respectively. The concd petrol extract gave a ppt. which was filtered and recrystallized from $CHCl_3$ to give **6** (64 mg). The residual extract (4.6 g) was repeatedly subjected to CC over silica gel (230–400 mesh) eluting with petrol (40–60°), followed by petrol–EtOAc mixtures and EtOAc to give a series of compounds which were purified by PTLC using toluene, toluene–EtOAc (9:1; 8:2) and $CHCl_3$–MeOH (98:2) to give: **1** (206 mg), **2** (14 mg), **3** (38 mg), **4** (4 mg), **5** (137 mg), **6** (60 mg), **7** (19 mg), **8** (6 mg), **9** (38 mg) and **10/11** (35 mg). The concd $CHCl_3$ extract (6 g) was subjected to VLC over silica gel eluting with petrol, petrol–EtOAc mixtures, EtOAc and EtOAc–MeOH mixtures to give 4 frs labelled A–D. Fr. A was CC over silica gel, eluting with toluene and toluene–EtOAc mixtures. The early fractions after PTLC (toluene–EtOAc, 9.5:0.5) gave **1** (15 mg). Fr. B was similarly treated and after PTLC (toluene–EtOAc, 8:2) gave **5** (72 mg), **6** (34 mg) and **7** (30 mg). Frs C and D gave ppts which were filtered and recrystallized from $CHCl_3$ to give **10/11** (553 mg). Further PTLC of the supernatant using $CHCl_3$–MeOH (95:5) gave more **10/11** (94 mg), **12** (5 mg) and **13** (14 mg).

2,5-Dihydroxy-7-methoxy-8-methlyflavanone and 2,5-dihydroxy-7-methoxy-6-methylflavanone **10** *and* **11** (*in* 1:0.94 *mixture*). Pale yellow crystalline powder; mp 134–136°; $[\alpha]_D$ +20.7 ($c = 0.105$, $CHCl_3$); UV: λ_{max}: 277, 334 (sh) nm; IR ν_{max} cm^{-1}: 3346 (OH), 2916, 1639 (C=O), 1448, 1317, 1205, 1155, 1126, 767; HR-EIMS: *m/z* (rel. int.): 300.1006 [M^+] (100) (calcd 300.0998), 283 (41), 282 (99), 281 (30), 154 (23), 181 (16), 77 (11); ^1H NMR and ^{13}C NMR (Table 2).

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REFERENCES

1. Le Thomas, A., in *Flore du Gabon*, Vol. 16, ed. A. Aubreville. Paris, 1969, p. 240.
2. Perry, L. M., *Medicinal Plants of East and Southeast Asia: Attributed Properties and Uses*, MIT Press, U.S.A., 1980, p. 20.
3. Kiang, A. K., Douglas, B. and Morsingh, F., *Journal of Pharmacy and Pharmacology*, 1960, **13**, 98.
4. Richomme, P., Sinbandhit, S., David, B., Hadi, A. H. A. and Bruneton, J., *Journal of Natural Products*, 1990, **53**, 294.
5. Achenback, H. and Hermrich, H., *Phytochemistry*, 1991, **30**, 1265.
6. Kodpinid, M., Sadavongvivad, C., Thebtaranonth, C. and Thebtaranonth, Y., *Phytochemistry*, 1984, **23**, 199.
7. Fráter, G. and Müller, U., *Helvetica Chimica Acta*, 1989, **72**, 653.
8. Weyerstahl, P., Schneider, S., Marshall, H. and Rustaiyan, A., *Liebigs Annals of Chemistry*, 1993, 111.
9. Agrawal, P. K., *^{13}C NMR of Flavonoids*. Elsevier, Tokyo, 1989.
10. Mayer, R., *Phytochemistry*, 1990, **29**, 1340.
11. Mabry, T. J., Markham, K. R. and Thomas, M. B., *The Systematic Identification of Flavonoids*. Springer, New York, 1970.
12. Burke, B. and Nair, M., *Phytochemistry*, 1986, **25**, 1427.
13. Chantrapromma, K., Pakawatchai, C., Skelton, B. W., White, A. H. and Worapatamasri, S., *Australian Journal of Chemistry*, 1989, **42**, 2289.
14. Chadenson, M., Hauteville, M. and Chopin, J., *Journal of The Chemical Society, Chemical Communications*, 1972, 107.
15. Byrne, L. T., Cannon, J. R., Gawad, D. H., Joshi, B. S., Skelton, B. W., Toia, R. F. and White, A. H., *Australian Journal of Chemistry*, 1982, **35**, 1851.
16. Williams, A. H., *Chemistry in Industry* (*London*), 1967, 1526.
17. Chopin, J., Hautville, M., Joshi, B. S. and Gawad, D. H. (1978) *Phytochemistry*, 1918, **17**, 332.

Bufadienolides and Other Constituents of *Urginea sanguinea*

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Abstract: **Fresh bulbs of *Urginea sanguinea* yielded stigmasterol, phloroglucinol, phloroglucinol 1-β-D-glucopyranoside (phlorin), scillaren A, a novel compound 5α-4,5-dihydroscillaren A (1), salicylic acid, and 3-hydroxy-4-methylbenzoic acid. The latter two showed weak antibacterial activity. The compounds were identified using spectroscopic techniques such as 1D and 2D NMR, EI-MS and FAB-MS.**

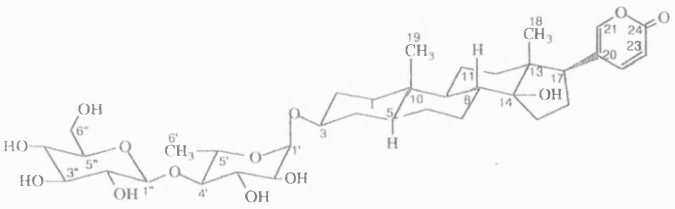
Urginea sanguinea (Schinz) Jessop (*Drimia sanguinea* Schinz) Liliaceae (Hyacynthaceae) is common in Southern Africa, with preference for hilly areas (1). The bulb is red and bleeds when cut, hence the name *sanguinea*. This species had not been investigated phytochemically until recently (2) but *U. sanguinea* is known to be among the most important poisonous plants hazardous to livestock in South Africa (2). After ingestion of the leaves, cattle, sheep, and goats show symptoms typical of cardiac glycoside poisoning (3). Tswana herbalists and traditional doctors use *U. sanguinea* to treat venereal diseases (1).

The ^{1}H- and ^{13}C-NMR (Table 1) spectra of 1 indicated a glucose, a rhamnose and a steroid nucleus. Several structural features were evident from the 400 MHz ^{1}H-NMR spectrum of 1. Clearly defined signals at δ = 6.27 (d, J = 9.7 Hz, H-23), 7.43 (d, J = 2.1 Hz, H-21) and 7.98 (dd, J = 2.1 and 9.7 Hz, H-22) are characteristic of protons constituting the δ-pyrone ring (4). The nature and stereochemistry of glycosyl moieties viz α-L-rhamnosyl and β-D-glucosyl were determined by anomeric proton resonances, respectively, at δ = 4.83 (d, J = 1.4 Hz, H-1′ and 4.57 (d, J = 7.8 Hz, H-1″). The steroid nucleus showed two methyls, eight methines, nine methylenes, and five quaternary carbons. The structure determination and assignments for 1 were achieved using COSY, HC-direct, HMBC, HOHAHA techniques and these data were consistent with the structure of 1 being a bufadienolide, a class of compounds which is very common in the genus *Urginea* (5–7). Most proton signals from rhamnose and glucose units were all superimposed and overlapped greatly. To resolve this signals HOHAHA was used to locate the individual spin systems of these sugar residues.

Table 1 ^{13}C-NMR (δ_C, 100 MHz) data for scillaren A and 1.

Carbon atom	Scillaren A*	1**
1	31.9	38.5
2	26.8	30.5[b]
3	72.6	77.5
4	120.7	35.3
5	146.4	45.7
6	34.9	30.6[b]
7	28.3[a]	28.9
8	41.5	43.0
9	49.5	51.4
10	37.0	37.1
11	20.9	22.7
12	39.7	41.9
13	47.9	49.2
14	83.0	86.1
15	28.5[a]	33.3
16	28.4[a]	30.2[b]
17	49.9	52.4
18	16.6	17.5
19	18.3	12.9
20	122.4	125.2
21	149.3	150.6
22	147.4	149.5
23	114.2	115.5
24	161.4	165.0
1′	99.0	99.6
2′	70.4	72.8
3′	70.5	72.6
4′	82.2	83.8
5′	67.0	68.6
6′	17.7	18.3
1″	105.5	105.9
2″	74.5	76.2
3″	76.6	78.2
4″	70.0	71.6
5″	77.0	78.3
6″	61.2	62.9

* DMSO-d_6.
** CD_3OD.
[a–b] assignments in each column interchangeable.



1

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Complete assignment of the sugar protons was done using HC-direct (Table 2), HMBC (Table 2), and HOHAHA (Table 3). The order in which the two sugars were attached to the aglycone was determined by HMBC which showed a correlation between H-3 (δ_H = 3.52–3.59) and the rhamnose anomeric carbon, C-1′ (δ_C = 99.6, d). This connectivity was further confirmed by an HMBC between the rhamnose anomeric proton (δ_H = 4.83) and the aglycone C-3 (δ_C = 77.5, d). The rhamnose was therefore attached directly to the C-3 of the aglycone moiety. The sequence of sugar residues was also determined by HMBC which showed a cross peak between the rhamnose H4′ proton (δ_H = 3.58–3.63) and the glucose anomeric carbon (δ_C = 105.9, d). This was further confirmed by another HMBC cross peak between the glucose anomeric proton (δ_H = 4.57) and the rhamnose C-4′ (83.3, d) carbon.

Table 2 HC-direct and HMBC correlations for **1**.

Protons	Carbon		
^1H	^1J	^2J	^3J
7.98	149.5 d		150.6 d, 165.0
7.43	150.6 d	125.2 s	52.4 d, 149.5 d, 165.0 s
6.29	115.5 d	165.0 s	125.2 s
2.54 t	52.4 d	30.2 t, 49.2 s, 125.2 s	41.9 t, 86.1 s, 149.5 s, 150.6 d
0.71 (Me)	17.5 q	49.2 s	41.9 t, 52.4 d, 86.1 s
1.64–1.70	30.2 t	52.4 d	49.2 s, 86.1 s
1.67–1.75; 1.32–1.36	22.7 t	41.9 t	43.0 d
1.59	43.0 d	86.1 s	49.2 t
0.82 (Me)	12.7 q	37.1 s	38.5 t, 45.7 d, 51.4 d
0.96–1.00	51.4 d		
1.06–1.13	45.7 d		12.7 q
3.52–3.59 m	77.2 d		99.6 d*
4.83 d	99.6 d	72.8 d	68.8 d, 72.6 d, 77.5 d
3.75	72.8 d	72.6 d	83.8 d
3.57–3.59	83.8 d	68.8d	18.3 q, 72.8 d, 105.9 d*
1.31 d	18.3 q	68.8 d	83.8 d
4.57 d	105.9 d		83.3 d*
3.21–3.23	76.2 d	78.2 d, 105.9 d	
3.27–3.28	78.2 d		78.3 d
3.34–3.35	71.6 d		76.2 d
3.30–3.31	78.3 d		78.2 d
3.68–3.70	62.9 t	78.3 d	
3.83–3.86	62.9 t	78.3 d	

* Key HMBC correlations.

The assignment of the aglycone moiety was done using HMBC, HC-direct and HOHAHA. The carbons which could not be assigned using the above methods were assigned using literature assignments for related compounds (5). The FAB-MS confirmed the molecular weight and the sugar sequence. The spectrum gave a peak at *m/z* 533 indicating the loss of a glucose residue and another peak at *m/z* 387 showing the loss of a glucose followed by the loss of a rhamnose moiety. The above information led to the determination of the structure of **1** as 14-hydroxybufa-20,22-dienolide-3-O-α-L-rhamnopyranosyl-4′-β-D-glucopyranoside (5α-4,5-dihydroscillaren A) which has not been previously reported.

The antibacterial activity of the crude water extract, the *n*-BuOH fraction and pure compounds are shown in Table 4. Salicylic acid is a well known antibacterial and preservative. However, it only showed activity at a loading of 100 µg/disc. Phenolic compounds like phloroglucinol which are expected to show antibacterial activity are not active at this loading but phloroglucinol derivatives show impressive activity against the same microorganisms (8, 9). Other phenolic compounds which show good activity are gallic acid (10) and caffeic acid esters (11). The activities of the isolated compounds compare very well with those of two antibacterial flavonoid glycoside derivatives tiliroside and platanoside (12). The toxic components (bufadienolides) make the plant unsafe to use it as a medicinal plant.

Table 3 HOHAHA correlations for **1** at 30 ms spin lock time.

^1H proton	HOHAHA correlation (30 ms)
H1′	H2′
H2′	H3′, H4′, H5′
H3′	H2′, H4′, H5′, Me6′
H4′	H3′, H4′, H5′, Me6′
H5′	H3′, H4′, Me6′
Me6′	H3′, H4′, H5′
H1″	H2″, H3″, H4″
H2″	H1″, H3″, H4″
H3″	H1″, H2″, H4″, H5″, H6″A, H6″B
H4″	H1″, H2″, H3″, H5″, H6″A, H6″B
H5″	H1″, H2″, H3″, H4″, H6″A, H6″B
H6″A, H6″B	H2″, H3″, H4″, H5″
H17	H16α, H16β, H15β
H16α	H17, H16β, H15β
H16β	H17, H16α, H15β
H15α	H15β
H15β	H17, H16α, H16β, H15α
H21	H23, H22
H22	H23, H21
H23	H22, H21

Table 4 Antibacterial activity of *Urginea sanguinea* and its constituents compared to chloramphenicol.

Organism	Compound	Loading (µg/disc)	Zone of inhibition (mm)
S. aureus	chloramphenicol	10	18
	chloramphenicol	25	20
	chloramphenicol	50	25
	crude water extr*	500	9
	n-butanol fr**	100	10
	salicylic acid	100	7.5
	3h-4-m-b	100	7
B. subtilis	chloramphenicol	10	20
	chloramphenicol	25	25
	chloramphenicol	50	31
	crude water extr*	500	8
	n-butanol fr**	100	9
	salicylic acid	100	8
	3h-4-m-b***	100	≤ 7

* Extr = extract.
** fr = fraction.
*** 3h-4-m-b = 3-hydroxy-4-methylbenzoic acid.

Materials and Methods

M.p.'s uncorrected: Kofler Hot Stage. *NMR*: on Bruker AMX 400; ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz), in either DMSO-d_6 or CD_3OD. ^{1}H- and ^{13}C-NMR chemical shifts were referenced to the residual solvent signals. *MS*: HREI AEI-MS 902 (70 eV) or a VG ZAB-E FAB MS. *IR*: Mattson Genesis Series FT-IR or a Perkin-Elmer 781, KBr discs. *UV spectra*: in CH_3OH on CE 505 UV Spectrophotometer. *Specific rotations* $[\alpha]_D$: Polatronic-D.

U. sanguinea bulbs were collected in Kgale, 10 km south of Gaborone, in May 1993, identified by Drs. L. Turton and B. Hargreaves and a voucher specimen (USRM 001) was placed at the National Herbarium, Gaborone, Botswana. *Extraction*: bulbs (3 kg) were chopped in pieces, extracted with cold water, concentrated, then freeze-dried to give 200 g residue. The crude water extract (195 g) was dissolved in distilled water (400 ml) and partitioned successively between 4 × 300 ml aliquots each of *n*-hexane, EtOAc, and *n*-BuOH. The extracts were evaporated *in vacuo* to yield 0.5, 1.5, and 120 g crude fractions, respectively. The residual water extract was freeze-dried to give 74 g of solid. The fractions were each tested (in triplicate) for antibacterial activity at a loading of 500 and 100 μg/disc using the disc diffusion assay (13, 14). *Separation*: The *n*-butanol extract (100 g) was added to a polyamide (300 g) column and eluted in a step-wise manner with toluene, toluene/EtOH (1 : 1), EtOH, EtOH/MeOH (1 : 1), MeOH, MeOH/$(CH_3)_2CO$ (1 : 1) and $(CH_3)_2CO$. Twenty-two fractions (150 ml each) were collected and the first seven fractions gave fatty acids and stigmasterol (50 mg).

TLC analyses of fractions 8–13 showed two major compounds and two minor ones. These fractions were purified on 100 g polyamide column and eluted with EtOH, EtOH/MeOH (1 : 1), MeOH, MeOH/H_2O (1 : 1) and H_2O in that order. The two major compounds were found in the first four (100 ml each). HPLC of these using a semi-preparative column (RP-18, MeOH/H_2O 68 : 32, flow rate 10 ml/min) led to isolation of scillaren A (65 mg), [t_R = 10 min] and 5α-4,5-dihydroscillaren A (26 mg), [t_R = 11.2 min].

Fractions 14–17 were put on Sephadex LH-20 (10 g, eluted with MeOH/$CHCl_3$, 1 : 1) and fractions containing the three minor components were further purified on HPLC (conditions same as above) to yield 3-hydroxy-4-methylbenzoic acid (6 mg) [t_R = 12.1 min] and salicylic acid (5 mg) [t_R = 13.4 min]. Fractions 18–22 showed two major spots which gave a bright red colour on spraying with 1% vanillin in sulphuric acid and heating. These were put on Sephadex G-10 (MeOH/H_2O 1 : 1). Ten fractions (50 ml each) were collected. Fractions 3–6 to yielded pure phlorin (78 mg) and fractions 8–10 gave phloroglucinol (24 mg).

5α-4,5-Dihydroscillaren A (**1**): Needles (from methanol), m.p. 192–194 °C, $[\alpha]_D$: −44.4 (*c* 0.12, MeOH) and formula $C_{36}H_{54}O_{13}$. UV λ_{max} (MeOH) nm: 298. IR υ_{max} (KBr) cm^{-1}: 3394, 2931, 1712, 1636, 1541, 1450, 1064. ^{1}H-NMR (400 MHz in CD_3OD): δ_H (*J* values in brackets) = 0.71 3H s, 0.82 3H s, 1.31 d (6.2), 2.05–2.08 m, 2.16–2.18 m, 2.54 t, 3.21–3.23 m, 3.27–3.28 m, 3.30–3.31 m, 3.34–3.35 m, 3.37 d, 3.57–3.59 m, 3.61 m, 3.68–3.70 dd (12.0, 4.2), 3.75 s, 3.83–3.86 dd (12.0, 1.6), 4.57 d (7.8), 4.83 d (1.4), 6.27 (9.7), 7.43 (2.1), 7.98 (2.1, 9.7). ^{13}C-NMR (see Table 1). FAB-MS: 717 (M + Na)$^+$, 695 (M + H)$^+$, 533 (M + H − 162 (glucosyl))$^+$, 387 (M + H − 162 − 146 (rhamnosyl))$^+$, 369 (aglycone + H − H_2O)$^+$, 351.

Scillaren A: Needles (from methanol), m.p. 270 °C dec., $[\alpha]_D$: −68.6 (*c* 0.82, MeOH). UV, IR, NMR and MS data agreed well with literature data (5, 6).

Stigmasterol: (^{1}H-NMR, MS, R_f, colour reaction with vanillin sulphuric acid) matched those of an authentic sample of stigmasterol.

Phloroglucinol: Brown amorphous powder, m.p. 117 °C. UV, IR, NMR and MS consistent with literature data (15).

Phlorin: Brown amorphous powder, m.p. 231–233 °C, $[\alpha]_D$: −71.0 (*c* 0.10, MeOH). UV, IR, NMR and MS data showed good agreement with literature data (15).

Salicylic acid: Needles, m.p. 158–160 °C. Spectral data were consistent with literature data (16).

3-Hydroxy-4-methylbenzoic acid: Needles, m.p. 215 °C (dec). UV λ_{max} (MeOH): 250, 272, 278 sh. IR υ_{max} (KBr): 3422, 2920, 1706, 1630, 1529, 1449, 1236, 1064. ^{1}H-NMR (400 MHz in CD_3OD): δ_H = 2.19 3H s, 7.09 d (8.2), 7.19 dd (2.2, 8.2), 7.49 d (2 Hz). ^{13}C-NMR (100 MHz in CD_3SOCD_3): δ_C 16.2 q, 115.4 d, 120.3 d, 129.5 s, 129.6 s, 130.3 d, 155.4 s, 167.8 s.

Microorganisms and antibacterial assay: The test organisms consisted of *Staphylococcus aureus* (NCTC 6751), *Bacillus subtilis* (NCTC 8326), *Escherichia coli* (NCTC 9001) and *Pseudomonas aeruginosa* (NCTC 6750). The agar disc diffusion assay (13, 14) was used to screen the crude water extract, the fractions and purified compounds. Paper discs (6 mm diameter) containing 500 and 100 μg of sample were placed on agar plates inoculated with test organisms. Experiments were carried out in triplicate and discs of standard antibiotic (chloramphenicol) were used as a positive control. The presence of a clear zone of inhibition after 18 hours was taken as a positive result.

References

[1] Hedberg, I., Staugard, F. (1989) Traditional Medicinal Plants, Ipeleng Publishers, Gaborone pp 117–118.
[2] Joulbert, J. P. J., Schultz, R. A. (1982) J. S. Afr. Vet. Assoc. 53, 25–28.
[3] Krenn, L., Kopp, B., Bamberger, M., Brustmann, E., Kubelka, W. (1993) Nat. Prod. Lett. 3, 139–143.
[4] Anderson, L. A. P., Steyn, P. S., van Heerden, F. R. (1984) J. Chem. Soc., Perkin Trans. I, 1573–1575.
[5] Krenn, L., Ferth, R., Robien, W., Kopp, B. (1991) Planta Med. 57, 560–565.
[6] Krenn, L., Jambrits, M., Kopp, B. (1988) Planta Med. 227–231.
[7] Kopp, B., Unterlungauer, M., Robien, W., Kubelka, W. (1990) Planta Med. 56, 193–197.
[8] Rios, J. L., Recio, M. C., Villar, A. (1990) Planta Med. 56, 646–647.
[9] Noble, H. M., Sidebottom, P. J., Lane, S. J., O'Neill, M. J. (1990) Planta Med. 56, 647–648.
[10] Majinda, R. R. T., Motswaledi, M., Waigh, R. D., Waterman, P. G. (1997) Planta Med. in press.
[11] Scholz, E., Heinrich, M., Hunkler, D. (1990) Planta Med. 56, 648–649.
[12] Mitrokotsa, D., Mitaku, S., Demetzos, C., Harvala, C., Mentis, A., Perez, S., Kokkinopoulos, D. (1993) Planta Med. 59, 517–520.
[13] Berghe, D. A. V., Vlietinck, A. J. (1991) Methods in Plant Biochemistry, Vol. 6, 47–69.
[14] Rios, J. L., Recio, M. C., Villar, A (1988) J. Ethnopharmacol. 23, 127–149.
[15] Foo, L. Y., Karchesy, J. (1989) Phytochemistry 28, 1237–1240.
[16] Pouchet, C. J., Behnke, J. (eds.) (1993) The Aldrich Library of ^{13}C and ^{1}H FT-NMR Spectra. 2, 1071A–1071A.

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NOVEL PRENYLATED ACETOPHENONES FROM *BOSISTOA SELWYNII*

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Abstract--From the aerial parts of *Bosistoa selwynii* (Rutaceae) four novel prenylated acetophenones were isolated and identified as pyranoselwynone (5-hydroxy-6-acetyl-{6,6-dimethyl-5ξ-hydroxy-4,5-dihydropyrano}-[3,2-h]-2,2-dimethylbenzopyran), selwynone (5,7-dihydroxy-6-acetyl-8-(3-methylbut-2-enyl)-3,4-dihydro-2,2-dimethyl-3ξ-hydroxypyran), furanoselwynone (5-hydroxy-6-acetyl-{5-[1-hydroxy-1-methylethyl]-4,5-dihydro-furano}-[3,2-h]-2,2-dimethylpyran), and isofuranoselwynone (7-hydroxy-8-acetyl-{5-[1-hydroxy-1-methylethyl]-4,5-dihydrofurano}-[3,2-f]-2,2-dimethylpyran). Other compounds isolated were a series of eight acridone alkaloids (reported on previously), the coumarins scopoletin and braylin and the triterpenes lupeol and β-amyrin. The structures of the new acetophenones were elucidated on the basis of NMR spectral data.

Keywords:--*Bosistoa selwynii*; Rutaceae; Prenylated Acetophenones; Selwynone; Pyranoselwynone; Furanoselwynone; Isofuranoselwynone.

INTRODUCTION

In a continuation of our investigation of the chemistry of the genus *Bosistoa* (Rutaceae)[1-6] we have undertaken a study of the aerial parts of *B. selwynii* Hartley, a tree found in the rain forests of south-east Queensland and north-east New South Wales.[7] In a preliminary report on this species[4] we presented evidence for the occurrence of prenylated acridone alkaloids identical to those found in *Bosistoa transversa*.[3] In this paper we report the isolation and identification of non-alkaloidal compounds; coumarins, pentacyclic triterpenes and four novel prenylated acetophenones.

RESULTS AND DISCUSSION

By a combination of vacuum liquid chromatography (VLC), gel filtration and PTLC procedures the hexane and ethyl acetate extracts of the leaf material of *B. selwynii* sample Hartley 15172 afforded the simple coumarin scopoletin and the common pentacyclic triterpenes lupeol and β-amyrin. In addition two novel acetophenones were obtained which were identified, primarily by NMR and MS as **1** and **2**, and given the trivial names selwynone and pyranoselwynone.

Selwynone gave, by HREIMS, a molecular ion at *m/z* 320 which solved for the empirical formula $C_{18}H_{24}O_5$, with a major fragment for M^+-C_4H_7, which is characteristic of a 3-methylbut-2-enyl substituent. The ^{1}H NMR spectrum (Table 1) confirmed the presence of the 3-methylbut-2-enyl moiety, an H-bonded phenolic proton, another aromatic phenol, an acetyl methyl and signals for two further methyls, probably geminal, and an ABX system -CH_2-CH(O)-. The ^{13}C NMR spectrum (Table 1) revealed signals for an aromatic ring with a phloroglucinol oxygenation pattern

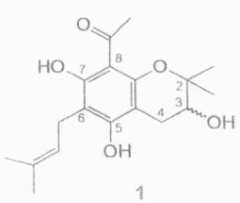
and an acetyl carbonyl. Direct H-C bonding was established by means of an HC-COBI experiment.[8]

Table 1. ^{1}H and ^{13}C NMR Chemical Shift Data (400 MHz, $CDCl_3$) and Selected ^{2}J and ^{3}J H-C Couplings for Selwynone, 1.

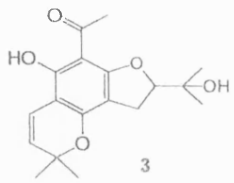
Position	δH (*J*, Hz)	δC	^{2}J	^{3}J
2		78.3		
2-Me	1.35 s	25.0	78.3	22.2, 69.9
2-Me	1.40 s	22.2	78.3	25.0, 69.9
3	3.80 t (5.8)	69.9		97.8
4	2.59 dd (16.8, 5.8) 2.87 dd (16.8, 5.8)	22.1	97.8	154.4, 160.3
4a		97.8		
5(OH)	6.30 s		160.3	97.8
5		160.3		
6		105.5		
7(OH)	14.07 s		162.7	105.5, 106.0
7		162.7		
8		106.0		
8a		154.4		
1'	3.40 d (7.4)	26.1	105.5, 122.0	136.6, 160.3, 162.7
2'	5.27 tq (7.4, 1.4)	122.0	26.1	
3'		135.5		
3'-Me(E)	1.68 d (1.4)	26.0	136.6	18.1, 122.0
3'-Me(Z)	1.61 s	18.1	136.6	26.0, 122.0
C=O		203.5		
COMe	2.60 s	33.6	203.5	106.0

On the basis of these data selwynone must be a 1,3,5-trioxygenated acetophenone with two free hydroxyls, one adjacent to the acetyl, a 3-methylbut-2-enyl group and a cyclised prenyl group which appears to be a 2,2-dimethyldihydropyran carrying further oxygenation. The positions of the various substituents were established through an HMBC experiment[8] which revealed ^{2}J and ^{3}J H-C couplings. Notably ^{3}J couplings from H_2-1' of the 3-methylbut-2-enyl side chain established

that it was located between the two hydroxyls on the aromatic ring. Similarly ^{3}J couplings from the geminal methyl groups and H_2-4 of the dihydropyran ring confirmed its placement. From the HMBC experiment the resonance position of all signals in the ^{13}C NMR spectrum could be unambiguously assigned. Selwynone must therefore have structure **1**, the stereochemistry of the secondary hydroxyl has not been established.



1



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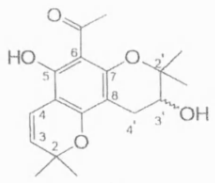

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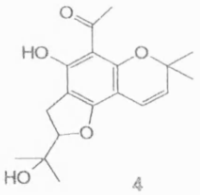

4

A second acetophenone from the extract analysed for *m/z* 319 (M+H) by FAB mass spectroscopy, solving for $C_{18}H_{22}O_5$. The ^{1}H NMR spectrum (Table 2) revealed the H-bonded hydroxyl, acetyl methyl and 3-hydroxy-2,2-dimethyldihydropyran ring seen in **1**. However, neither the second phenolic OH nor the 3-methylbut-2-enyl moiety were observed and were replaced by a 2,2-dimethylpyran. The ^{13}C NMR spectrum (Table 2) confirmed the same phloroglucinol oxygenation pattern and the HMBC (Table 2) substantiated the relative positions of the pyran and

dihydropyran rings. This compounds (**2**), which appears to be novel, has been given the trivial name pyranoselwynone.

Table 2 ^{1}H and ^{13}C NMR Chemical Shift Data (400 MHz, $CDCl_3$) and Selected ^{2}J and ^{3}J H-C Couplings for Pyranoselwynone, **2**.

Position	δH	δC	^{2}J	^{3}J
2		78.4		
2-Me_2	1.45 s	28.7	78.4	125.2
3	5.45 d (10.0)	125.2	78.4	102.6
4	6.65 d (10.0)	116.4	102.6	78.4, 158.1, 160.4
4a		102.6		
5(OH)	14.05 s		160.4	102.6, 105.8
5		160.4		
6		105.8		
7		155.9		
8		98.4		
8a		158.1		
2'		78.8		
2'-Me	1.35 s	25.2	78.8	22.1, 68.9
2'-Me	1.40 s	22.1	78.8	25.2, 68.9
3'	3.80 t (5.8)	68.9		98.4
4'	2.55 dd (17.0, 5.8)	25.7	68.9, 98.4	78.8, 155.9, 158.1
	2.85 dd (17.0, 5.8)			
C=O		203.4		
COMe	2.60 s	33.5	203.4	105.8

A second sample of *B. selwynii* (Hartley 15176) yielded pyranoselwynone, scopoletin, lupeol and β-amyrin but did not contain selwynone. In addition it yielded the common furoquinoline alkaloid skimmianine, the pyranocoumarin braylin and a mixture of two further novel acetophenones. The HREIMS of the mixture suggested a single molecular ion, solving for $C_{18}H_{22}O_5$ with a major fragment at M-C_3H_7O indicative of a hydroxyisopropanyl group. The ^{1}H NMR of the mixture,

run in acetone-d_6, revealed several features (H-bonded OH, acetyl, 2,2-dimethylpyran) in common with **2**. The remaining signals consisted of an ABX system with different coupling constants to the dihydropyran ring of **1** and **2**, and resonances for two methyls (in each component of the mixture) between δ 1.35 and 1.22. While paucity of material did not allow us to obtain an adequate ^{13}C NMR spectrum the H-detected HMBC experiment revealed that these methyl resonances showed ^3J coupling to a carbon at *ca.* δ 93, which is typical for C-2 oxymethine carbon of a dihydrofuran.[9] Other couplings observed from the HMBC experiment confirmed that these acetophenones shared the same oxygenation pattern as **1** and **2**.

The remaining problem was to identify the positions of the pyran and dihydrofuran rings in the two components. To do this the ^1H NMR spectrum of **2** was re-run in acetone-d_6. This revealed that the resonances for the pyran olefinic protons had identical chemical shift in **2** and in the major constituent of the mixture which must therefore have structure **3**, to which we assign the trivial name furanoselwynone. The minor component of the mixture must be **4**, which we call isofuranoselwynone.

The chemistry of *B. selwynii* is striking in that it produces a series of acridone alkaloids very similar to those of *B. transversa*.[3] By contrast the previous study on *B. transversa* failed to reveal the presence of either acetophenones or coumarins.

EXPERIMENTAL

Mps uncorr. UV: MeOH; IR: KBr or $CHCl_3$; EIMS: direct probe insertion (90-130°C) at 70 eV. FABMS: VG ZAB-E with nitrobenzyl alcohol matrix. NMR: run in $CDCl_3$ or acetone-d_6. NOESY and HMBC experiments were obtained on a Bruker AMX-400 instrument using standard Bruker microprograms.

Plant material. Hartley 15172, collected 24th October 1991, Wide Bay District, State Forest 220, Malmaison, Queensland; Hartley 15176, collected 24th October 1991, Wide Bay District, Didcot, Stony Creek, Queensland. Both vouchers are deposited at the Australian National Herbarium, Canberra.

Extraction and isolation of non-alkaloidal compounds. The ground leaves of the two samples (TGH-15172, 120 g; TGH-15176, 180 g) were separately extracted (Soxhlet) with petroleum ether (b.p. 60-80°C), then EtOAc, and finally MeOH. The petrol extract of TGH-15172 was concentrated under reduced pressure to give 5.5 g of solid. Part of this (2 g) was fractionated by column chromatography with Sephadex LH-20 eluting with $CHCl_3$, followed by PTLC of the fractions with silica gel (solvent $CHCl_3$:EtOAc, 19:1). This procedure yielded β-amyrin (40 mg), lupeol (3 mg), **1** (3.5 mg), scopoletin (11 mg) and **2** (3.5 mg). Similar treatment of 2 g of the petrol extract of TGH-15176 (total yield 7 g) gave braylin (6 mg), **1** (4 mg), a mixture of **3** and **4** (3 mg), and scopoletin (2 mg).

Identification of known compounds. Skimmianine: - Identical (UV, IR, NMR, MS) with sample reported previously.[10] *Scopoletin* - Identical (UV, IR, NMR, MS) with commercial sample.[11] *β-Amyrin* - Identical (UV, IR, NMR, MS) with commercial sample.[11] *Lupeol* - Identical (UV, IR, NMR, MS) with sample.[12] *Braylin* – Amorphous, Found: M+ 258.0883; $C_{15}H_{14}O_4$ requires 258.0892; UV λmax (MeOH) nm: 261, 270, 350 nm, IR νmax (KBr) cm^{-1}: 1720, 1565, 1285, 1150, ^1H NMR (400MHz, $CDCl_3$) δ 7.57 (d, *J* 9.4 Hz, H-4), 6.77 (s, H-5), 6.26 (d, *J* 9.4 Hz, H-3), 6.24 (d, *J* 10.0 Hz, H-4'), 5.74 (d, *J* 10.0 Hz, H-3'), 3.90 (s, 6-OMe), 1.53 (s, 2'-Me_2), EIMS *m/z* (rel. int.): 258 $[M]^+$ (38), 263 (100), 228 (16).

Pyranoselwynone (**1**): Amorphous, $[\alpha]_D$ +16.2° (c. = 0.004, $CHCl_3$), UV λmax (MeOH) nm (log ε): 213 (4.15), 295 (3.82), 350 (3.47), IR νmax ($CHCl_3$) cm^{-1}: 3446, 2930, 1598, 1432, 1365, 1283, 1192, 1126, 1101, 996, 887, 734, NMR – see Table 2, FABMS m/z (rel. int.): 319 $[M+H]^+$ (80), 318 (100), 303 (60), 247 (45).

Selwynone (**2**): Amorphous, $[\alpha]_D$ +15° (c. = 0.001, $CHCl_3$), UV λmax (MeOH) nm (log ε): 215 (4.02), 295 (4.21), 345 (3.28), IR νmax ($CHCl_3$) cm^{-1}: 3380, 2925, 1611, 1429, 1368, 1269, 1227, 1088, 757, NMR – see Table 1, EIMS m/z (rel. int.): 320 $[M]^+$ (70), 305 (10), 265 (100), 247 (67), 218 (42), 193 (53).

Furanoselwynone and Isofuranoselwynone (**3**, **4**): M+ = 318.1489, UV λmax (MeOH) nm: 212, 269, 300, 348, IR νmax (KBr) cm^{-1}: 3420, 2928, 1616, 1435, 1365, 1288, 1198, 1103, 1012, 756, ^1H NMR (400MHz, acetone-d_6) δ: 13.86, 13.55 (2 x OH – H-bonded), pyran ring protons, 6.56, 5.55 (ABq, *J* 10.1 Hz) and 6.41, 5.53 (ABq, *J* = 9.9 Hz), pyran ring methyls, 1.51, 1.49, 1.44, 1.43, dihydrofuran ABX system, 3.01 (dd, J 15.0, 8.1 Hz), 2.95 (dd, J 15.0, 8.1 Hz), 4.85 (t, J 8.1 Hz), and 3.01 (dd, J 15.0, 8.1 Hz), 2.95 (dd, J 15.0, 8.1 Hz), 4.81 (t, J 8.1 Hz), isopropyl methyls, 1.35, 1.35, 1.28, 1.22, acetyl methyls, 2.61, 2.59, EIMS m/z (rel. int.): 318 (37), 303 (100), 300 (25), 244 (45).

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REFERENCES

1. I. C. Parsons, A. I. Gray, and P. G. Waterman, (1993) New triterpenes and flavonoids from the leaves of *Bosistoa brassii Journal of. Natural Products* **56**, 46.
2. A. A. Auzi, A. I. Gray, T. G. Hartley, R. D. Waigh, and P. G. Waterman, (1994) A xanthine alkaloid from the leaves of *Bosistoa floydii. Phytochemistry* **36**, 535.
3. A. A. Auzi, T. G. Hartley, R. D. Waigh, and P. G. Waterman, (1996) Acridone alkaloids from *Bosistoa transversa. Phytochemistry* **42**, 235.
4. A. A. Auzi, R. D. Waigh, and P. G. Waterman, (1994) Acridone alkaloids from *Bosistoa selwynii. Journal of. Pharmmacy and Pharmacology,* Supplement **46**, 1086.
5. A. A. Auzi, T. G. Hartley, R. D. Waigh, and P. G. Waterman, (1996) Flavonoids from *Bosistoa medicinalis.* In Flavonoids and Bioflavonoids: Proceedings of the International Bioflavonoid Symposium, Vienna, 1995, ed. S. Antus, M. Gabor, K. Vetschera, Akademiai Kiado, Budapest, pp. 227-230.
6. A. A. Auzi, T. G. Hartley, R. D. Waigh, and P. G. Waterman, (1997) Phloroglucinol derivatives from leaves of *Bosistoa pentacocca. Phytochemistry,* **45**, 1673.
7. T. G. Hartley, (1977) A revision of the genus *Bosistoa* (Rutaceae). *Journal of the Arnold Arboretum,* **58**, 426.
8. for a description of the NMR methods used, see: A. Quader, A. I. Gray, P. G. Waterman, C. Lavaud, G. Massiot and I. H. Sadler (1991) Phebaloparvilactone: a new protolimonoid from the leaves of *Phebalium squamulosum* ssp. *parvifolium. Tetrahedron* **47**, 3611.
9. Breitmaier, E. and Voelter, W. (1987) ^{13}C NMR Spectroscopy, Methods and Applications, 3rd. Edn., Verlag Chemie GmbH, Weinheim.
10. F. Fish, and P. G. Waterman, (1971)Chloroform-soluble alkaloids of *Fagara leprieurii. Phytochemistry* **10**, 3322.
11. Purchased from Apin Chemicals Milton Park, Abingdon, U.K. (scopoletin – no. 1371c, β-amyrin – no. 7155a).
12. S. M. Zhong, P. G. Waterman, and J. A. D. Jeffreys, (1984) Naphthoquinones and triterpenes from African *Diospyros* species. *Phytochemistry* **23**, 1067.

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A NOVEL RETRODIHYDROCHALCONE FROM THE STEM BARK OF *UVARIA MOCOLI*

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Key Word Index—*Uvaria mocoli*; Annonaceae; chalcones; flavanones; dihydrochalcones; oxoaporphine alkaloids.

Abstract—Investigation of the ethyl acetate extract of the stem bark of *Uvaria mocoli* resulted in the isolation of the novel retrodihydrochalcone 2-hydroxy-4,5,6-trimethoxydihydrochalcone together with the known flavonoids 2′-hydroxy-4′,6′-dimethoxychalcone, 2′-hydroxy-4′,5′,6′-trimethoxychalcone, 5,7-dimethoxyflavanone and 5,7,8-trimethoxyflavanone, the oxoaporphines lysicamine, liriodenine and isomoschatoline, benzoic acid and a mixture of sitosterol and stigmasterol. All the compounds were identified by analysis of their spectral data.

INTRODUCTION

The genus *Uvaria* L. (Annonaceae) comprises some 150 species [1]. About 20 of these have so far been investigated and have yielded a wide range of secondary metabolites with interesting biological activities [2], including sesquiterpenes [3, 4], indole derivatives [5, 6], 1-benzyltetrahydroisoquinoline derivatives [1, 7], benzyl benzoate esters [8], cyclohexene epoxides [9, 10], flavonoids [11, 12] and acetogenins [13, 14]. We have examined the stem bark of *Uvaria mocoli* De Wild. & Th. Dur., a large woody climber which grows in the tropical forest zone of west Africa between Sierra Leone and Zaire [15], and in this paper report the isolation of five flavonoids, three oxoaporphines, benzoic acid and a mixture of sitosterol and stigmasterol. Among the flavonoids is the novel retrodihydrochalcone 2-hydroxy-4,5,6-trimethoxydihydrochalcone (**3**).

RESULTS AND DISCUSSION

The ethyl acetate extract of the stem bark of *U. mocoli*, through a series of chromatographic fractionations, yielded the flavonoids **1**–**5**, the oxoaporphines alkaloids **6**–**8**, benzoic acid and a mixture of sitosterol and stigmasterol. The ^{1}H NMR spectra of **1** and **2** showed the presence of an unsubstituted B-ring and the characteristic *trans*-propenone moiety and a 2′-hydroxylated ring A of a chalcone [16]. Compound **1** further showed signals for two aromatic methoxyl groups and two protons *meta*-coupled to each other, which identified it as 2′-hydroxy-4′,6′-dimethoxychalcone [17]. Compound **2** differed in showing a single aromatic proton and three methoxyl singlets. It was identified as 2′-hydroxy-4′,5′,6′-trimethoxychalcone [18] rather than 2′-hydroxy-3′,5′,6′-trimethoxychalcone, on the basis of the ^{13}C NMR chemical shift values for the methoxyl carbons (δ 62.1, 61.5, 56.3) which revealed that two of them were sterically hindered by being flanked by substituents in both *ortho* positions [20, 21].

The ^{1}H NMR spectra of **4** and **5** showed the typical AMX system of flavanones [16] and an unsubstituted ring B. Based on the spectral characteristics they were identified as 5,7-dimethoxyflavanone [19] and 5,7,8-trimethoxyflavanone [20], respectively. Evidence for the substitution pattern of **5** was again derived from the ^{13}C NMR spectrum which revealed that only one of the methoxyl groups was sterically hindered (δ 61.7, 56.5, 56.3), whereas for the 5,6,7-methoxylation pattern two would be hindered.

The novel compound **3** was isolated as a yellow-brown amorphous solid. The UV spectrum showed maxima at 228, 275 and 307 nm, suggesting an extended benzyl chromophore and the IR spectrum showed hydroxyl and carbonyl absorptions. The HREI mass spectrum gave a molecular ion at *m/z* 316.1186 which solved for $C_{18}H_{20}O_5$. The ^{1}H NMR spectrum (Table 1) indicated the presence of an unsubstituted aromatic ring, an aromatic proton singlet, a broad proton singlet for a hydroxyl group, three

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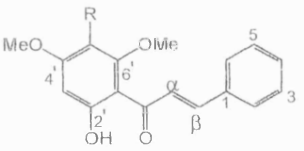
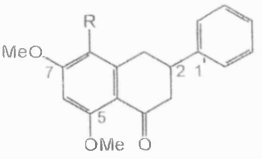
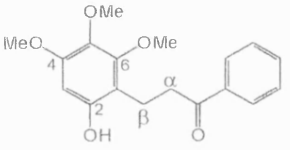


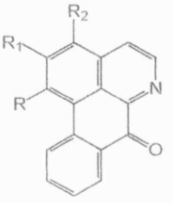
	R
1	H
2	OMe



	R
4	H
5	OMe



3



	R	R_1	R_2
6	OMe	OMe	H
7	-O-CH_2-O-		H
8	OMe	OMe	OH

Table 1. ^{1}H and ^{13}C NMR data ($CDCl_3$) and some significant ^2J and ^3J correlations in the HMBC spectrum of **3**

Position	δ_H*	δ_C†	$^2J_{H,C}$	$^3J_{H,C}$
1		113.5		
2		151.2		
3	6.35 *s*	97.7	δ 151.2 (C-2), δ 152.8 (C-4)	δ 113.5 (C-1), δ 136.0 (C-5)
4		152.8		
5		136.0		
6		152.3		
C=O		203.3		
C-α	3.43 *m*	17.5	δ 29.9 (C-β), δ 203.3 (C=O)	δ 113.5 (C-1), δ 136.1 (C-1′)
C-β	2.95 *m*	29.9	δ 17.5 (C-α), δ 113.5 (C-1)	δ 151.2 (C-2), δ 152.3 (C-6), δ 203. (C=O)
1′		136.3		
2′/6′	7.99 *m*	128.8		
3′/5′	7.44 *m*	128.6		
4′	7.59 *m*	134.0		
2-OH	8.45			
4-OMe	3.80	55.9		δ 152.8 (C-4)
5-OMe	3.78	61.0		δ 136.0 (C-5)
6-OMe	3.95	61.0		δ 152.3 (C-6)

* Solutions were referenced to $CHCl_3$ at δ 7.27 (400 MHz).
† Solutions were referenced to $CHCl_3$ at δ 77.23 (100 MHz).

methoxyls and two methylene multiplets. The ^{13}C NMR spectrum (Table 1) revealed six aromatic methines, five of which contributed to the unsubstituted phenyl ring, six quaternary aromatic carbons, a carbonyl at δ 203.3, two methylenes and three methoxyl signals. These data suggested a dihydrochalcone structure for **3**.

Examination of the unsubstituted aromatic moiety revealed that the two aromatic proton multiplets *ortho* to the ring junction (H-2′/H-6′) are relatively deshielded (δ 7.99) compared to those of compounds **1** (δ 7.61) and **2** (δ 7.66), suggesting the inductive effect of an adjacent C=O function and hence a benzoyl partial structure in **3**. This was confirmed by the ion at *m/z* 105 in the EI mass spectrum. Thus, **3** was a dihydrochalcone with substitution in ring B and not in ring A. In the ^{13}C NMR spectrum, two equivalent methoxyl carbons resonated at δ 61.0, with the other at δ 55.9, suggesting that the latter must be placed *ortho* to a proton while the former two, which are both sterically hindered, must be flanked by the ring junction and/or *ortho* oxygenated functions [20, 21]. This suggested that **3** was either 2-hydroxy-4,5,6-trimethoxydihydrochalcone or 4-hydroxy-2,5,6-trimethoxydihydrochalcone. The structure was established from 2D NMR experiments including HMBC and NOESY.

In the HMBC spectrum (Table 1) the position of the carbonyl function was confirmed by the ^2J correlation to H_2-α and the ^3J correlations to H_2-β and H-2′/6′ protons. The three methoxyl singlets showed ^3J correlations to the signals at δ_C 136.0 (C-5), δ_C 152.8 (C-4) and δ_C 152.3 (C-6), leaving the other oxygenated carbon at δ 151.2 (C-2) for the hydroxyl substitution. The aromatic proton singlet showed ^2J correlations to one methoxyl-bearing carbon (C-4) and the hydroxyl-bearing carbon (C-2), and ^3J correlations to the ring junction carbon (C-1) and the shielded methoxyl-bearing carbon (C-5). The H_2-β also showed ^2J correlation to the same ring junction carbon and ^3J correlations to the hydroxyl-bearing carbon (C-2) as well as a methoxyl-bearing carbon (C-6). This established unambiguously the position of the OH at C-2 rather than C-4. In the NOESY experiment, an nOe correlation was observed between the protons of the 4-OMe and H-3, and H_2-α and H-2′/6′. Thus, **3** was identified as 2-hydroxy-4,5,6-trimethoxydihydrochalcone.

2-Hydroxy-4,5,6-trimethoxydihydrochalcone (**3**) belongs to the retro-dihydrochalcone group of flavonoids because of the phloroglucinol-like oxygenation pattern of ring A [22]. It has been shown by feeding experiments that these retrochalcones are formed through carbonyl transposition of the corresponding normal chalcones, so that ring A is derived from cinnamoyl CoA and ring B, from the acetate-malonate pathway [22, 23]. Consequently, the co-isolation of **3** with **2** is significant, as **2** could serve as the normal chalcone intermediate which undergoes 1,3-carbonyl transposition to give **3**. This is the first report of a retrodihydrochalcone in the Annonaceae but the retrochalcone, tepanone (2-hydroxy-3,4,6-trimethoxychalcone) has been isolated from the root and stem bark of *Ellipeia cuneifolia* [24] and the stem bark of *U. pandensis* [25].

Alkaloids **6**–**8** were also identified by analysis of their spectral data and comparison with those published as the known oxoaporphines lysicamine [26], liriodenine [26] and isomoschatoline [27] respectively. This is, surprisingly, the first report of oxoaporphine alkaloids in *Uvaria*.

EXPERIMENTAL

General

Mps: uncorr; UV: MeOH; IR: $CHCl_3$; ^1H and ^{13}C NMR: recorded on Bruker AMX-400 instrument; MS: AEI-MS 902 double focusing instrument with direct probe insert at 70 eV; Si gel CC and VLC: Merck 60, 230–400 mesh; Petrol is petroleum ether (bp 60–80°).

Plant material

The plant materials used for this study were collected from Gyakye (Ashanti Region) in Ghana by the Herbarium Unit of the Forestry Department, Kumasi, Ghana, and identified by comparison with herbarium specimens.

Extraction and isolation of compounds

The stem bark powder (0.5 kg) of *U. mocoli* was Soxhlet extracted with EtOAc and concentrated under reduced pressure to give 10.2 g of dry extract. VLC fractionation of the EtOAc extract (10 g) over Si gel, eluting with toluene, toluene-$CHCl_3$ mixtures, $CHCl_3$ and finally $CHCl_3$–MeOH mixtures, yielded four frs: A (up to 5% $CHCl_3$ in toluene), B (up to 10% $CHCl_3$ in toluene), C (up to 20% $CHCl_3$ in toluene), D (up to 20% MeOH in $CHCl_3$). Fr A was CC over Si gel, eluting with petrol and increasing the polarity with EtOAc; frs 28-41 were bulked together and subjected to *prep*-TLC (toluene : EtOAc 9 : 1) to give **1** (118 mg). Fraction B was treated as in fr A and frs 21–31 after *prep*-TLC gave **2** (41 mg). Fraction C was CC over Si gel and eluted with petrol, petrol–EtOAc mixtures and EtOAc; frs 16–20 after *prep*-TLC (petrol : EtOAc, 8 : 2) gave sitosterol/stigmasterol (12 mg); frs 23–31 on *prep*-TLC (petrol : EtOAc, 8 : 2) yielded **3** (32 mg); frs 58–80 were further CC over Si gel using petrol–EtOAc mixtures and frs 13–24 yielded **4** (99 mg). The rest of the frs collected were pooled and further fractionated on *prep*-TLC (petrol : EtOAc, 8 : 2) to give benzoic acid (3 mg), **4** (11 mg) and **5** (9 mg). Fr D was VLC over Si gel and eluted with 10% $CHCl_3$ in petrol and increasing the polarity till 20% MeOH in $CHCl_3$. Frs 7-8 (from $CHCl_3$: MeOH 95 : 5) was fractionated by *prep*-TLC (petrol : EtOAc, 8 : 2) to give **6** (35 mg)

and 7 (12 mg). Fr 10 (10% MeOH in $CHCl_3$) was CC over Si gel and **8** was collected as a blue band (4 mg).

Benzoic acid

Amorphous precipitate, identity confirmed by co-TLC with authentic sample.

Sitosterol/Stigmasterol

Needle crystals, identity confirmed by co-TLC with authentic samples isolated in our laboratory.

2′-Hydroxy-4′,6′-dimethoxychalcone (**1**)

Yellow amorphous solid, mp 92–94° (lit. 91.5–92°, [17]), $[M]^+$ 284.1041 ($C_{17}H_{16}O_4$ requires 284.1049), UV, IR, ^1H NMR, ^{13}C NMR, MS, in agreement with published data [17].

2′-Hydroxy-4′,5′,6′-trimethoxychalcone (**2**)

Yellow amorphous solid, mp 99–101° (lit. 102° [18]); $[M]^+$ 314.1144 ($C_{18}H_{18}O_5$ requires 314.1154), UV, IR, ^1H NMR, ^{13}C NMR, MS, in agreement with published data [18].

2-Hydroxy-4,5,6-trimethoxydihydrochalcone (**3**)

Brown-yellow amorphous compound; Found: $[M]^+$ 316.1186 ($C_{18}H_{20}O_5$ requires 316.1311), UV λ_{max} (MeOH) 228, 275, 307 (*sh*) nm; IR ($CHCl_3$, cm^{-1}) 3332 (OH) 1668 (C=O); EIMS *m/z* (rel. int., %) 316 (44) (M^+), 301 (75), 197 (44) 184 (35), 153 (545), 105 (100), 77 (95).

5,7-Dimethoxyflavanone (**4**)

White amorphous solid, mp 166–166° (lit. 169–172°, [19]), $[M]^+$ 284.1044 ($C_{17}H_{16}O_4$ requires 284.1049), UV, IR, ^1H NMR, ^{13}C NMR, MS, in agreement with published data [19].

5,7,8-Trimethoxyflavanone (**5**)

White amorphous solid, mp 151–152° (lit 154° [20]), $[M]^+$ 314.1144 ($C_{18}H_{18}O_5$ requires 314.1154), UV, IR, ^1H NMR, ^{13}C NMR, MS, in agreement with published data [20].

Lysicamine (**6**)

Yellow amorphous solid; $[M]^+$ 291.0892, ($C_{18}H_{13}O_3N$ requires 291.0895); UV, IR, ^1H NMR, MS, in agreement with published data [26].

Liriodenine (**7**)

Yellow amorphous solid; $[M]^+$ 275.0582, ($C_{17}H_9NO_3$ requires 275.0582), UV, IR, ^1H NMR, MS, in agreement with published data [26].

Isomoschatoline (**8**)

Dark red amorphous powder; $[M]^+$ 307.0803, ($C_{18}H_{13}NO_4$ requires 307.0845), UV, IR, ^1H NMR, MS, in agreement with published data [27].

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REFERENCES

1. Leboeuf, M., Cavé, A., Bhaumik, P. K., Mukherjee, B. and Mukherjee, R., *Phytochemistry*, 1982, **21**, 2783.
2. Parmar, V. S., Tyagi, O. D., Malhotra, A., Singh, S. K., Bisht, K. S. and Jain, R., *Natural Product Reports*, 1994, 219.
3. Muhammad, I. and Waterman, P. G., *Journal of Natural Products*, 1988, **51**, 719.
4. Weenen, H., Nkunya, M. H. H., Mgani, Q. A., Psathmus, M. A., Waibel, R. and Achenbach, H., *Journal of Organic Chemistry*, 1991, **56**, 5865.
5. Achenbach, H. and Raffelsberger, B., *Tetrahedron Letters*, 1979, 2571.
6. Muhammad, I. and Waterman, P. G., *Journal of Natural Products*, 1985, **48**, 571.
7. Panichpol, K., Waigh, R. D. and Waterman, P. G., *Phytochemistry*, 1977, **16**, 621.
8. Kodpinid, M., Sadavongvivad, C., Thebtaranonth, C. and Thebtaranonth, Y., *Phytochemistry*, 1984, **23**, 199.
9. Jolad, S. D., Hoffmann, K. H., Cole, J. R., Tempesta, M. S. and Bates, R. B., *Phytochemistry*, 1984, **23**, 935.
10. Nkunya, M. H. H., Weenen, H., Bray, D. H., Mgani, Q. A. and Mwasunbi, L. B., *Planta Medica*, 1991, **57**, 341.
11. Hufford, C. D. and Oguntimein, B. O., *Journal of Natural Product*, 1982, **45**, 337.
12. Nkunya, M. H. H., Achenbach H., Renner, C., Waibel, R. and Weenen, H., *Phytochemistry*, 1990, **29**, 1261.
13. Jolad, S. D., Hoffmann, J. J., Schram, K. H., Cole, J. R., Tempesta, M. S., George, R. K. and Bates, R. B., *Journal of Organic Chemistry*, 1982, **47**, 3151.
14. Chen, Y. and Yu, D. Q., *Journal of Natural Products*, 1996, **59**, 507.
15. Le Thomas, A., *Fam.* 16, *Annonacees* in *Flore du Gabon*, ed. A. Aubreville. Museum National d'Histoire Naturelle, Paris, 1969, p. 43.
16. Mabry, J. T., Markham, K. R. and Thomas, M. B., *The Systematic Identification of Flavonoids*. Springler-Verlag, New York, 1970.

17. Itokawa, H., Morita, M. and Mihashi, S., *Phytochemistry*, 1981, **20**, 2503.
18. Bohlmann, F., Mahanta, P. K. and Zdero, C., *Phytochemistry*, 1978, **17**, 1935.
19. Bick, I. R. C., Brown, B. B. and Hills, W. E., *Australian Journal of Chemistry*, 1972, **25**, 449.
20. Panichpol, K. and Waterman, P. G., *Phytochemistry*, 1978, **17**, 1363.
21. Makriyannins, A. and Fesik, S., *Journal of the American Chemical Society*, 1982, **104**, 6462.
22. Saitoh, T. and Shibata, S., *Tetrahedron Letters*, 1975, 4461.
23. Ayabe, S. and Furaya, T., *Journal of Chemical Society Perkin Transactions*, 1982, **1**, 2725.
24. Colegate, S. M., Din, L. B., Ghisalberti, E. L. and Latiff, A., *Phytochemistry*, 1992, **31**, 2123.
25. Nkunya, M. H. H., Waibel, R. and Achenbach, H., *Phytochemistry*, 1993, **34**, 853.
26. Guinaudeau, H., Leboeuf, M. and Cave, A., *Journal of Natural Products*, 1975, **38**, 275
27. Atti, S. A. E., Ammar, H. A., Phoebe, C. H. Jr., Schiff, P. L. Jr. and Slatkin, D. J., *Journal of Natural Products*, 1981, **45**, 476.

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Isobutylamides and Coumarins from *Melicope melanophloia*

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Keywords: *Melicope melanophloia*; Rutaceae; isobutylamides; coumarins; chemotaxonomy

1. Subject and source

Aerial parts of the Australian species *Melicope melanophloia* C.T. White (Rutaceae), a tree 3 m high growing in dry rocky rain forest bordering a creek, were collected from Stony Creek, near Didcot, Wide Bay District, southeastern Queensland. A voucher specimen (TGH 15173) was deposited at the Australian National Herbarium, Canberra.

2. Previous work

The isolation of the furanocoumarin isopimpinellin has been reported previously (Jones et al., 1968).

3. Present study

The *n*-hexane extract of the dried, ground aerial parts of *M. melanophloia* (500 g) was subjected to VLC (eluting with *n*-hexane and EtOAc in mixtures of increasing

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polarity). Fractions were collected and combined according to composition and subjected to gel filtration chromatography (Sephadex LH-20 eluting with $CHCl_3$) and PTLC (*n*-hexane: EtOAc 3 : 2, $CHCl_3$: EtOAc 19 : 1). All the compounds isolated were identified using ^1H and ^{13}C NMR along with various 2D NMR experiments (e.g. COSY, HMBC, NOESY), and HREIMS, comparing data with literature values where available. The compounds isolated and identified were the furanocoumarins xanthotoxin (19 mg), bergapten (18 mg) and isopimpinellin (15 mg) along with the angular pyranocoumarin alloxanthoxyletin (4 mg) and the simple coumarin limettin (3 mg). In addition, the isobutylamide fagaramide (30 mg) and the closely related 3,4-dimethoxy-N-(2′-methylpropyl)-phenylpropylamide (4 mg), the latter previously reported from the family Piperaceae (Achenbach *et al.*, 1986), were also isolated.

4. Chemotaxonomic significance

The presence of coumarins is relatively common throughout the family Rutaceae. The occurrence of isobutylamides, however, is much rarer, being confined to the proto-rutaceous species *Zanthoxylum* and *Tetradium* (Ng *et al.*, 1987) whilst the structurally similar amides of isovaleric and senecioic acids have been found from some *Glycosmis* species (Greger et al., 1996). The taxonomic placements of the genera *Euodia* and *Melicope* are currently under review (Hartley, 1997) with *Melicope melanophloia* to be placed into a new genus. Some *Euodia* species have been transferred to the closely related proto-rutaceous genus *Tetradium* (Hartley, 1981). The presence of isobutylamide compounds in *M. melanophloia*, therefore, suggests that this plant may well have a close affiliation to the proto-rutaceous genera of *Tetradium* and *Zanthoxylum*.

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References

Achenbach, H., Fietz, W., Worth, J., Waibel, R., Portecop, J., 1986. Planta Med. 12.
Greger, H., Zechner, G., Hofer, O., Vajrodya, S., 1996. J. Nat. Prod. 59, 1163.
Hartley, T.G., 1981. Gard. Bull. Singapore 34, 91.
Hartley, T.G., 1997. personal communication.
Jones, S.R., Lamberton, J.A., Price, J.R., Sioumis, A.A., 1968. Aust. J. Chem. 21, 3079.
Ng, K.M., But, P.P.H., Gray, A.I., Hartley, T.G., Kong, Y.C., Waterman, P.G., 1987. Biochem. Syst. Ecol. 15, 587.

614 *J. Nat. Prod.* **1998,** *61,* 614−619

Novel and Insecticidal Isobutylamides from *Dinosperma erythrococca*

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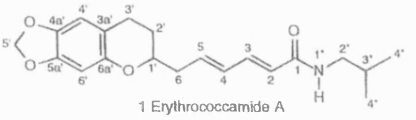
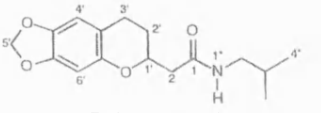
Through insecticidal bioassay-guided fractionation and isolation, five isobutylamides **1**−**5** were isolated from *n*-hexane and EtOAc extracts of the aerial parts of *Dinosperma erythrococca* (Rutaceae). The structures of compounds **1**−**5** were established through 1D and 2D NMR. Compounds **1**−**3** were identified as erythrococcamides A−C and represent two novel classes of isobutylamide. Compounds **4**−**5** were identified as *N*-(2-hydroxy-2-methylpropyl)-6-phenyl-2(*E*),4(*E*)-hexadienamide (**4**)[1,2] and *N*-(2-methylpropyl)-6-phenyl-2(*E*),4(*E*)-hexadienamide (**5**),[1,3] respectively. Compound **1** showed lethal activity against the housefly (*Musca domestica*) (kill EC_{50} = 20.2 ppm) and the tobacco budworm (*Heliothis virescens*) (kill EC_{50} = 74.2 ppm). Compound **4** showed lethal activity against *H. virescens* at 500 ppm, while compound **5** showed lethal activity against *M. domestica* at 500 ppm.

The Australian rainforest tree, formerly known as *Melicope erythrococca* F. Muell. (Rutaceae), has recently been reassigned to a new genus and renamed *Dinosperma erythrococca* (F. Muell.) T. G. Hartley.[4] Previous screening of this species had indicated that an aqueous extract of the seeds had weak insecticidal activity against the milkweed bug (*Oncopeltus fasciatus*).[5] Further examination had shown that a $CHCl_3$ extract of the aqueous extract of the aerial parts had moderate antifeedant activity against the black carpet beetle (*Attagenus piceus*); however, no followup work on these observations has since been reported.[5] Previous phytochemical studies on *D. erythrococca* (as *M. erythrococca*) reported the isolation of the common triterpene lupeol and the phenylpropanoid compound elemicin.[6]

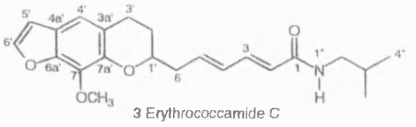
In ongoing work to find novel insecticidal compounds from natural sources, screening of an EtOH extract of *D. erythrococca* against six economically important insect species[7] suggested it to have slight insecticidal activity. In this paper, we report the bioassay-guided fractionation of *D. erythrococca* based on insecticidal activity, which led to the isolation and identification of five isobutylamides (**1**−**5**). All five compounds were either novel or had not previously reported from natural sources.



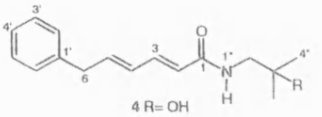
1 Erythrococcamide A



2 Erythrococcamide B



3 Erythrococcamide C



4 R= OH
5 R= H

Results and Discussion

Soxhlet extraction of the dried ground aerial parts sequentially with *n*-hexane, EtOAc, and MeOH, followed by screening of the extracts, showed the *n*-hexane and EtOAc extracts to have lethal activity against the housefly and the tobacco budworm at 10 000 ppm. Bioassay-guided fractionation of both extracts gave an active fraction from which compounds **1**−**3** were isolated. A polar fraction of the EtOAc extract also showed activity against the two-spotted spider mite. From this fraction compound **4** was isolated, although this compound was inactive against the mites when tested. Further investigation of the activity against the two-spotted spider mite was undertaken by extraction and bioassay-guided fractionation of the remaining wood of *M. erythrococca*. This led to the detection of a mite-active fraction in the *n*-hexane extract from which compound **5** was isolated.

From the ^1H and ^{13}C NMR and MS, compounds **1**−**5** could be identified as being isobutylamides (see Tables

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Table 1. ^{1}H NMR Spectral Data for Compounds **1**–**5** (in $CDCl_3$ at 400 MHz)

position	1	2	3	4	5
2	5.82 (d, J = 15.8 Hz)	2.58 (dd, J = 14.8, 7.9 Hz), 2.52 (dd, J = 14.8, 4.0 Hz)	5.81 (d, J = 15.0 Hz)	5.84 (d, J = 15.2 Hz)	5.80 (d, J = 15.3 Hz)
3	7.21 (dd, J = 15.8, 10.7 Hz)		7.24 (dd, J = 15.0, 10.6 Hz)	7.24 (m)	7.23 (m)
4	6.24 (dd, J = 15.2, 10.7 Hz)		6.30 (dd, J = 15.3, 10.6 Hz)	6.17 (dd, J = 15.2, 10 Hz)	6.15 (dd, J = 15.3, 9.6 Hz)
5	6.12 (dt, J = 15.2, 7.0 Hz)		6.19 (dt, J = 15.3, 7.0 Hz)	6.24 (dt, J = 15.1,6.2 Hz)	6.22 (dt, J = 15.3, 6.3 Hz)
6	2.57 (m), 2.46 (m)		2.56 (m), 2.73 (m)	3.49 (d, J = 6.2 Hz)	3.49 (d, J = 6.3 Hz)
1′	3.97 (m)	4.33 (dddd, J = 12.1, 7.9, 4.0, 2.2 Hz)	4.10 (m)		
2′	1.94 (m), 1.67 (m)	2.00 (m), 1.64 (m)	2.05 (m), 1.78 (m)	7.17 (br d, J = 7.1 Hz)	7.17 (br d, J = 6.9 Hz)
3′	2.72 (m), 2.64 (m)	2.65 (ddd, J = 16.4, 5.6, 3.2 Hz), 2.80 (ddd, J = 16.4, 10.2, 6.3 Hz)	2.93 (m), 2.89 (m)	7.31 (br t, J = 7 Hz)	7.31 (br t, J = 7 Hz)
4′	6.47 (s)	6.50 (s)	6.94 (s)	7.24 (m)	7.23 (m)
5′	5.84 (s)	5.86 (d, J = 1.4 Hz), 5.87 (d, J = 1.4 Hz)	6.61 (d, J = 2.2 Hz)		
6′	6.34 (s)	6.30 (s)	7.49 (d, J = 2.2 Hz)		
7′-OCH_3			4.10 (s)		
1″	5.70 (br s)	6.13 (br s)	5.48 (br t)	5.98 (br s)	5.58 (br s)
2″	3.16 (t, J = 6.7 Hz)	3.15 (dt, J = 13.4, 6.5 Hz), 3.08 (dt, J = 13.4, 6.5 Hz)	3.18 (t, J = 6.3 Hz)	3.35 (d, J = 6.1 Hz)	3.17 (t, J = 6.4 Hz)
3″	1.80 (n, J = 6.7 Hz)	1.80 (m)	1.80 (n, J = 6.4 Hz)	R= −OH, 1.71 (br s)	1.81 (n,J = 6.7 Hz)
4″	0.92 (d, J = 6.7 Hz)	0.93 (d, J = 6.7 Hz)	0.92 (d, J = 6.4 Hz)	1.24 (s)	0.93 (d,J = 6.7 Hz)

Table 2. ^{13}C NMR Spectral Data for Compounds **1**–**5** (in $CDCl_3$)

position	1	2	3	4	5
1	166.4	170.4	166.3	167.4	166.4
2	123.1	43.0	123.1	122.5	123.0
3	140.8		140.9	141.6	140.9
4	131.1		131.3	129.5	129.6
5	137.4		137.4	141.4	140.8
6	38.9		38.9	39.4	39.4
1′	75.1	73.3	75.7	139.2	139.3
2′	27.2	27.5	27.1	128.9	128.9
3′	24.8	24.7	25.2	128.8	128.8
3a′	113.3	113.4	119.6		
4′	108.3	108.5	114.2	126.6	126.6
4a′	141.5[a]	141.9[a]	122.1		
5′	100.9	101.1	106.4		
5a′	146.5[a]	146.7[a]			
6′	98.8	98.6	144.5		
6a′	149.4	148.7	146.1		
7′			133.7		
7a′			144.4		
7′-OCH_3			61.1		
2″	47.2	47.0	47.2	50.7	47.2
3″	28.8	24.7	28.8	71.3	28.8
4″	20.3	20.30, 20.29	20.3	27.5	20.3

[a] Assignments interchangeable.

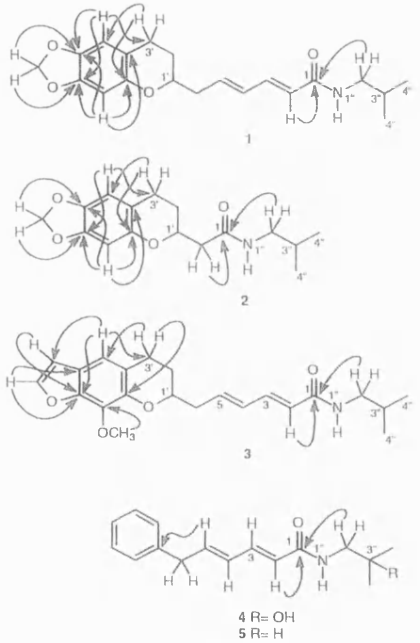


Figure 1. Important ^2J and ^3J H–C correlations seen in HMBC spectra of **1**–**5**.

1 and 2 for ^{1}H and ^{13}C NMR data, respectively). The IR spectrum of compound **1** suggested a secondary amide function (1662, 1567 cm^{-1}), while the mass spectrum gave the molecular formula $C_{20}H_{25}NO_4$ with a fragment indicative of an isobutylamide at M^{+} 72 (loss of *i*-BuNH through α-cleavage at carbonyl) and the appearance of fragments at 57 amu (*i*-Bu) and 100 amu (CONH-*i*-Bu).[8]

The ^{1}H NMR and ^{1}H–^{1}H COSY spectra of **1** gave signals for an isobutylamide group (δ 5.70 (H-1″), 3.16 (H_2-2″), 1.80 (H-3″), 0.92 (2 × H_3-4″)).[9–11] In addition, the signals of a conjugated diene moiety were apparent (δ 5.82 (H-2), 7.21 (H-3), 6.24 (H-4), 6.12 (H-5)) in a trans-trans configuration as indicated by the coupling constants of 15.8 and 15.2 Hz for each pair of olefinic protons.[11] The protons of the conjugated diene moiety could be linked together from the ^{1}H–^{1}H COSY spectrum and placed adjacent to the carbonyl carbon (C-1) from a ^2J HMBC correlation (Figure 1) from the olefinic proton H-2 to C-1. The last proton of the conjugated diene chain (H-5) showed coupling to a methylene group H_2-6 (δ 2.57, 2.46), which, in turn, coupled to a deshielded, oxygen-bearing methine H-1′ (δ 3.97). H-1′ exhibited further coupling to methylene protons H_2-2′ (δ 1.94, 1.67), which, in turn, showed coupling to methylene protons of H_2-3′ (δ 2.72, 2.64). From H_2-3′ no further

coupling was seen in the $^1H-^1H$ COSY spectrum, indicating that the carbon adjacent to H_2-3′ was quaternary. The ^{13}C resonances for the partial structures were assigned from the HC-COBI experiment.

Analysis of the aromatic region of the ^{13}C NMR spectrum showed the presence of six aromatic carbons. Four quaternary aromatic carbons were seen, C-3a′ (δ 113.3), C-4a′ (δ 141.5), C-5a′ (δ 146.5), and C-6a′ (δ 149.4), the latter three being deshielded due to bonding to oxygen atoms. Two aromatic methines were observed at δ 108.3 (C-4′) and 98.8 (C-6′). The lack of visible coupling between the two aromatic protons H-4′ (δ 6.47) and H-6′ (δ 6.34) and the presence of only one aromatic ring in the molecule suggested that the aromatic protons were para to one another. A methylenedioxy group (H_2-5′ δ 5.84, δ_C 100.9) could be identified from the 1D NMR spectra, while the HMBC experiment (Figure 1) revealed these protons to show ^3J correlations to the aromatic carbons C-4a′ (δ 141.5) and C-5a′ (δ 146.5).

The structure of compound **1** was finally determined from the HMBC experiment (Figure 1), which allowed the partial structures to be linked together. The attachment of the methylene C-3′ to the quaternary C-3a′ was shown through ^3J correlations of H-3′ to C-4′ and H-4′ to C-3′, thereby establishing C-3a′ as the non-oxygen-bearing quaternary carbon (δ 113.3). Two of the oxygen-bearing quaternary carbons were accounted for as part of the methylenedioxy ring. The remaining oxygen must be attached to C-6a′ (δ 149.4), bridging the gap between C-6a′ and the deshielded oxygen-bearing methine H-1′/C-1′ (δ_H 3.97, δ_C 75.1) to form a benzopyran moiety and give the final structure **1**. A NOESY experiment was carried out in an attempt to determine whether H-1′ was axial or equatorial, but no firm conclusions could be made from the NOE correlations seen.

The ^1H and ^{13}C JMOD spectra of compound **2** were very similar to those seen for **1**, showing the presence of the (methylenedioxy)benzopyran moiety and the isobutylamide function but lacking the conjugated diene. The $^1H-^1H$ COSY and HMBC experiments allowed the various partial structures to be linked together. The $^1H-^1H$ COSY spectrum showed a correlation from the methylene group H_2-2 (δ 2.58, 2.52) to the sp^3 oxymethine H-1′ (δ 4.33) of the pyran ring and from H-1′ to the adjacent methylene protons H_2-2′ (δ 1.64, 2.00) and then H_2-3′ in the pyran ring (δ 2.65, 2.80). From the HMBC experiment, the correlations shown by the H_2-2 protons confirmed that this methylene joined the pyran ring and the isobutylamide function, through ^2J correlations between H-2 and C-1 (δ 170.4) and C-1′ (δ 73.3) and a ^3J correlation to C-2′ (δ 27.5). Finally, the H-2″ protons showed a ^3J correlation to C-1 (δ 170.4), confirming the attachment of the isobutylamine function to the carbonyl group.

The ^1H and ^{13}C NMR spectra revealed compound **3** to also be similar to compound **1**. Similarities included a benzopyran moiety and the 2(*E*),4(*E*)-hexadieneisobutylamide group, with novel features being the presence of an aromatic methoxyl group and a furan moiety. In the ^{13}C NMR spectrum the methoxyl group resonated at δ 61.1, which indicated that the ortho positions were both substituted.[12] This suggested that the furan ring was in a linear configuration with respect to the benzopyran moiety. The HMBC experiment (Figure 1) allowed the piecing together of the molecule and final determination of the structure. The significant correlations came from the sole aromatic proton H-4′, which showed ^3J correlations with the methylene C-3′ (δ 25.2) and with the oxygen-bearing aromatic carbons at C-6a′ (δ 146.1) and C-7a′ (δ 144.4), the latter being part of the pyran ring. In addition, a ^3J correlation was seen between the aromatic proton H-4′ and the furan methine H-5′ at δ 106.4, which meant that the oxygen substituent of the furan ring was meta to the aromatic proton, which was further substantiated by the fact that the furan proton H-5′ was relatively shielded at δ 6.61. Had the methoxyl group been in the C-4′ position, then the H-5′ proton would have been deshielded to ca. δ 7 due to the peri-deshielding effect of the methoxyl. This is analogous to the ^1H NMR chemical shifts seen with the furanocoumarins bergapten (**6**) and xanthotoxin (**7**).[13]

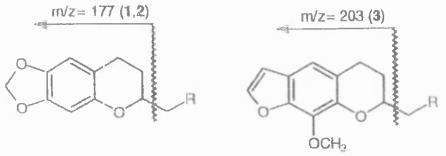
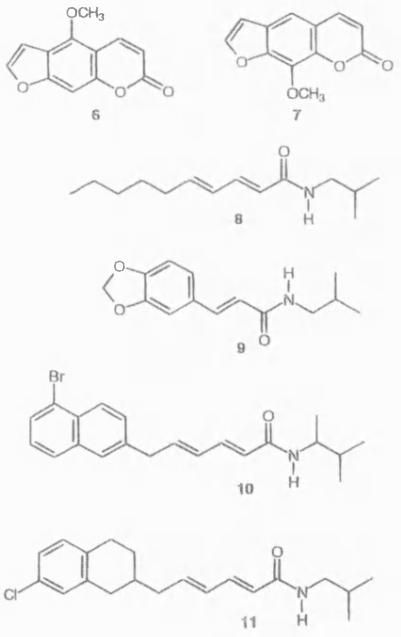


Figure 2. Probable major fragments seen from the mass spectra of compounds **1**–**3**.



The mass spectra of compounds **1**–**3** gave typical fragmentation patterns for isobutylamides as described above but also showed major fragments that could be ascribed to cleavage of the benzopyran moiety from the amide side chain (Figure 2).

Both the ^1H and ^{13}C NMR spectra showed compounds **4** and **5** to have the conjugated 2(*E*),4(*E*)-hexadiene

Table 3. Insecticidal Activity of Compounds 1–5 and 8–9

compd	insecticidal activity[a]	other observations
1	housefly kill EC_{50} = 20.2 ppm. rel potency = 3% of cypermethrin tobacco budworm kill EC_{50} = 74.2 ppm. Rel potency = 9.9% of profenofos	also showed housefly knockdown activity at 500 ppm
2	inactive at 500 ppm	
3	inactive at 500 ppm	
4	gave 80–100% kill against tobacco budworm at 500 ppm[b]	
5	housefly kill EC_{50} = 59.9 ppm.[c] Rel potency = 1.8% of cypermethrin[b]	also showed housefly knockdown activity at 500 ppm
8	inactive at 1000 ppm	also showed housefly knockdown activity at 2500 ppm
9	inactive at 2500 ppm	

[a] Tested against the six pest species listed in ref 7. [b] Due to shortage in compound, only preliminary activity-studies done. [c] Results are for the synthetic compound made during other research carried out by Zeneca Agrochemicals Ltd.[31]

system of **1** together with a monosubstituted aromatic ring. In **4**, an isobutylamide group was not immediately apparent. Two deshielded 2H doublets were observed (δ 3.35, 3.49) together with a deshielded 6H singlet (δ 1.24), the latter suggestive of two methyl groups attached to a quaternary oxygen-bearing carbon. In the ^{1}H–^{1}H COSY experiment, starting from H-2 (olefinic adjacent to carbonyl), it was possible to trace the conjugated diene function to H-5, which showed a correlation to a 2H doublet at δ 3.49 (H_2-6). The N–H proton meanwhile showed a correlation to the 2H doublet at δ 3.35 (H_2-2″). The JMOD ^{13}C NMR revealed the presence of two nonaromatic quaternary carbons, one of which could be identified as the carbonyl C-1 (δ 167.4) while the other (δ 71.3) must be the carbon to which the two methyl groups were attached and which is deshielded due to an OH substituent. The 2D HC-COBI allowed the assignment of direct ^1J C–H correlations, and HMBC (Figure 1) facilitated the complete assignment of the structure. The correlations of importance from the HMBC experiment showed attachment of the aromatic ring to the 2(*E*),4(*E*)-hexadiene chain as demonstrated by a ^3J correlation between H-5 and the aromatic quaternary C-1′ (δ 139.3). Similarly, the protons H_2-6 gave a ^3J correlation with the carbon C-2′. The protons H_2-2″ showed correlations to C-1, C-3″, and C-4″. Compound **4** was thus identified as *N*-(2-hydroxy-2-methylpropyl)-6-phenyl-2(*E*),4(*E*)-hexadienamide. Comparison of the physical and spectroscopic properties of compound **4** with those quoted in the literature[1,2] for the synthetic compound showed them to be identical.

Analysis of the ^{1}H and ^{13}C NMR spectra of compound **5** showed the same unsubstituted isobutylamide group as **1**–**3**, together with the phenyl-2(*E*),4(*E*)-hexadienamide unit of **4**. From 1D and 2D NMR experiments compound **5** was confirmed as *N*-(2-methylpropyl)-6-phenyl-2(*E*),4(*E*)-hexadienamide and showed physical and spectral properties identical to the synthetic compound reported.[1–3]

The insecticidal activities of compounds **1**–**5** are shown in Table 3 together with those of the isobutylamides pellitorine (**8**) isolated from *Zanthoxylum tessmannii*[14] and fagaramide (**9**) isolated from *Dinosperma melanophloia* (formerly *Melicope melanophloia*).[15] Various naturally occurring isobutylamides have been shown to be active against insect species.[16,17,20] Synthetic work on isobutylamides used compound **5** as a lead compound and structural variations led to improved activity, the most potent compounds being **10** and **11**.[3,18,19]

The results found in this work mirror the synthetic studies, with **1** being implicated as the major active compound of the crude extract. The active compounds all possessed conjugated 2(*E*),4(*E*)-hexadienamide moieties, which are crucial to activity.[3,18–20] The absence of the diene function in **2** and **9** resulted in a complete loss in activity. Compound **1** was shown to be more potent than **5** and to possess a broader range of activity. This suggested that substitution of the aromatic ring can lead to further increases in potency, a view substantiated by the synthetic work.[3,18,19] Compound **3** was inactive at 500 ppm on all screens, which suggested a limit to substitution on the aromatic ring above which total loss of activity is seen, possibly due to restricted access to the receptor site.

The close resemblance of the aromatic moiety of **3** to xanthotoxin (**7**) may possibly allow the plant to exploit two modes of insecticidal action. Furanocoumarins such as bergapten (**6**) and xanthotoxin (**7**) have been reported to have antifeedant and insecticidal activity,[21–24] and the presence of hexadienamide and furanocoumarin-like moieties in **3** could lead to a dual mode of action against pest species that *D. erythrococca* encounters in the wild. The substitution of **1** with a methylenedioxy moiety may enhance the activity of this compound through inhibition of metabolism, an activity characterized by the sesamin-type insecticide synergists.[25,26]

Compounds **4** and **5** came from mite active fractions but when tested did not show any activity against mites. These fractions were then recombined and tested, but activity against the two-spotted spider mite could not be reestablished. The isolation of two structurally very similar isobutylamides from fractions that showed activity against mites suggests that they may be implicated in the activity seen for the fractions. The possibility of synergism between the isobutylamides and compound(s) unknown (that are possibly unstable) cannot be entirely ruled out, although the lack of activity in the recombined fractions points to another compound being responsible for activity in the mite screen.

In an assay measuring relative potency, compound **1** possessed 3% of the activity of the pyrethroid insecticide cypermethrin against the housefly. It has been reported that **11** gave 40% of the activity of the pyrethroid permethrin against houseflies.[19] A general comparison of activity between permethrin and cypermethrin suggested that cypermethrin was approximately three to four times more potent than permethrin,[27] which in turn suggests that **1** has approximately 9–12% of the activity of permethrin.

The taxonomic placements of the genera *Euodia* and *Melicope* in the Rutaceae are currently under review.[28] Some northern *Euodia* species have been transferred to the closely related "proto-rutaceous" genus *Tetradium*.[29] For the family Rutaceae as a whole, the distribution of isobutylamides has been restricted to the two proto-rutaceous genera *Zanthoxylum* and *Tetradium*[30] and to the species *Dinosperma melanophloia* (formerly *Melicope melanophloia*),[15] which is to be placed into the newly reassigned genus *Dinosperma* along with *D. erythrococca* on morphological grounds.[4,28] This work represents the second report of the isolation of isobutylamides from this newly created genus. The compounds isolated in this study mark an advance in biosynthetic complexity for this class of compound as a whole, employing both cinnamate and acetate pathways (probably with mevalonate involvement in the formation of the furan ring) to yield more potent insecticidal compounds. The isobutylamides may also prove to be chemotaxonomic markers to identify other members of this genus.

Experimental Section

General Procedures. Melting points (uncorrected) were determined on a Kofler hot-stage apparatus. UV spectra were recorded using a Perkin-Elmer 552 spectrophotometer with the sample dissolved in MeOH. IR spectra were recorded as KBr disks on a Matson Genesis series FT-IR spectrophotometer. Optical rotations were measured on a Bellingham and Stanley ADP220 instrument and the samples dissolved in $CHCl_3$. HREIMS were run on a JEOL JMS-AX505HA double-focusing instrument at 70 Ev. EIMS were run on a JEOL DX303 in EI mode. NMR spectra (both one- and two-dimensional) were obtained on a Bruker AMX 400 (400 MHz for ^1H) spectrometer, all samples dissolved in $CDCl_3$. Vacuum−liquid chromatography (VLC) was carried out using Merck silica gel 60H. Column chromatography was carried out using silica gel (Merck 0.063−0.2 μm) or Sephadex LH-20 (Pharmacia). Preparative HPLC was performed using a Gilson 601 system with a Spherisorb ODS2 C-18 column (25 mm i.d. × 250 mm). Analytical and preparative TLC (PTLC) were performed on Merck silica gel 60 F_{254} (0.2 mm thick) and Merck silica gel 60 PF_{254} (1 mm thick) plates, respectively, and the plates visualized under UV (254 and 366 nm) and by spraying with 1% vanillin−H_2SO_4 and then heating.

Plant Material. Aerial parts of *D. erythrococca* (F. Muell.) T. G. Hartley were collected from rainforest (at 500 m altitude), Atherton Tableland, Cook District, Queensland. A voucher specimen (voucher no. Waterman 91/3) was deposited at the Australian National Herbarium, Canberra.

Testing for Insecticidal Activity. The initial screening involved counting out approximately 10 of the individual test species[7] into a container prepacked with an adequate food supply. The extract/fraction/compound was dissolved in a 50:50 mixture of acetone and an aqueous suspending agent, and the complete mixture was sprayed into the container holding the test species. After a period of time (different for each species, typically 3−5 days), the assay was assessed for percentage lethality and scored. The control experiment involved spraying a similar container holding insects and food supply with the same volume of carrier and assessing mortality after the same period of time. Crude extracts were screened at a concentration of 10 000 ppm with fractions being tested at 1000−5000 ppm. Pure active compounds in sufficient quantity were tested further at varying concentrations to ascertain EC_{50} values.

Extraction. The dried, ground plant material was extracted using a Soxhlet apparatus. The initial EtOH extract showed weak activity against the housefly and the tobacco budworm. Large-scale extraction was done sequentially with *n*-hexane followed by EtOAc and then MeOH. Two different extracts were prepared, one for all aerial parts, the second for the wood.

Bioassay-Guided Fractionation and Isolation. Results from the insecticidal bioassays showed the *n*-hexane and EtOAc extracts to possess lethal activity against the housefly and the tobacco budworm. The EtOAc extract also showed lethal activity against the two-spotted spider mite. VLC fractionation of the *n*-hexane extract on silica gel was carried out, eluting with *n*-hexane and then *n*-hexane with increasing amounts of EtOAc. Fractions were analyzed by TLC and similar fractions combined. The fractions were tested for biological activity, and the fraction F3 (eluted with 30%-70% EtOAc) was active against the housefly and the tobacco budworm. This fraction was then subjected to column chromatography using Sephadex LH-20, eluting with $CHCl_3$ and collecting 40 mL fractions. Fraction 2 again showed lethality against the housefly and the tobacco budworm. Fraction 2 was dissolved in 1:1 *n*-hexane/EtOAc, and a precipitate formed that was filtered off to give compound **1** (18 mg). The solution was subjected to PTLC (*n*-hexane/EtOAc 4:1) to give compounds **2** (8 mg) and **3** (4 mg).

The EtOAc extract showed lethal activity against the two-spotted spider mite, the housefly, and the tobacco budworm. VLC fractionation of the *n*-hexane extract on silica gel was carried out, eluting with *n*-hexane and then *n*-hexane containing increasing amounts of EtOAc, and finally with MeOH. The activity against the housefly and tobacco budworm could be attributed to the presence of **1**. The activity against the two-spotted spider mite was found to occur in a more polar fraction, eluted from the VLC column using 60% EtOAc in MeOH to 100% MeOH. The active fraction was twice subjected to column chromatography using Sephadex LH-20, eluting with $CHCl_3$ with increasing amounts of MeOH and collecting 40 mL fractions. The fractions were analyzed by TLC and like fractions combined and screened for activity. Compound **4** was isolated from the active fraction by preparative HPLC using a Gilson 601 system with a Spherisorb ODS2 C-18 column (25 mm i.d × 250 mm) and eluting with 40% MeOH in H_2O, collecting the major peak at 10.3 min. Compound **4** was inactive against mites when screened at a concentration of 2500 ppm. A reexamination of the mite activity was undertaken by extracting the remaining wood of *D. erythrococca* as above. The activity against mites was concentrated in the *n*-hexane extract. VLC of the *n*-hexane extract led to an active fraction eluted by 20−70% EtOAc in *n*-hexane. The active fraction was subjected to gel chromatography using Sephadex LH-20 eluting with $CHCl_3$ and collecting 40 mL fractions.

Fraction 2 was found to be active, and this was subjected to column chromatography using silica gel (eluting *n*-hexane/EtOAc). The mite-active fraction was eluted using 22–30% EtOAc in *n*-hexane. The major compound from this fraction was compound **5**, which precipitated out of a $CHCl_3$/MeOH mixture.

Erythrococcamide A (1) [6-[3,4-dihydro-6,7-(methylenedioxy)-2*H*-1-benzopyran-2-yl]-*N*-(2-methylpropyl)-2(*E*),4(*E*)-hexadienamide]: needles from hexane/EtOAc; mp 160–161 °C; $[\alpha]_D$ (*c* 0.01, $CHCl_3$) +133°; UV λ_{max} (MeOH) 257, 310 nm (sh); IR ν_{max} (KBr disk) 3391, 3283, 2959, 1662, 1635, 1621, 1567, 1513, 1486 cm^{-1}; EIMS *m/z* 343 [M^+] (62), 271 (6), 193 (23), 177 (100), 167 (55), 151 (42), 141 (11), 135 (24), 119 (18), 100 (12), 91 (17), 77 (12), 68 (23), 57 (58), 28 (47).

Erythrococcamide B (2) [2-[3,4-dihydro-6,7-(methylenedioxy)-2*H*-1-benzopyran-2-yl)-*N*-(2-methylpropyl]acetamide]: pale yellow solid; mp 95 °C; $[\alpha]_D$ (*c* 0.001, $CHCl_3$) +73°; UV λ_{max} (MeOH) 256 nm; IR ν_{max} (KBr disk) 3432, 2958, 1643, 1508, 1487, 1477 cm^{-1}; HREIMS 291.1468 (calcd for $C_{16}H_{21}NO_4$, 291.1471); EIMS *m/z* 291 [M^+] (100), 220 (18), 218 (25), 202 (10), 190 (14), 177 (32), 176 (23), 175 (24), 162 (36), 151 (56), 149 (42), 141 (20), 134 (23), 123 (28), 121 (30), 68 (59), 57 (70).

Erythrococcamide C (3)[6-(5,6-dihydro-9-methoxy-7*H*-furo[3,2-*g*]-1-benzopyran-7-yl)-*N*-(2-methylpropyl)-2(*E*),4(*E*))-hexadienamide]: pale yellow wax; mp 42 °C; $[\alpha]_D$ (*c* 0.001, $CHCl_3$) +185°; UV λ_{max} (MeOH) 255, 260, 270, 295 nm; IR ν_{max} (KBr disk) 3440, 3324, 2958, 1631, 1624, 1573, 1504,1496, 1478, 1296, 1153 cm^{-1}; HREIMS 369.1969 (calcd for $C_{22}H_{27}NO_4$, 369.1940); EIMS *m/z* 369 [M^+] (73), 345 (11), 343 (14), 291 (54), 267 (18), 256 (14), 218 (18), 203 (100), 177 (53), 176 (20), 167 (79), 151 (55), 57 (91).

***N*-(2-Hydroxy-2-methylpropyl)-6-phenyl-2(*E*),4(*E*)-hexadienamide (4):** yellow waxy solid; mp 77 °C; UV λ_{max} (MeOH) 257 nm; IR ν_{max} (KBr disk) 3440, 2876, 1658, 1627, 1550, 1460, 1377, 1257 cm^{-1}; EIMS *m/z* 259 [M^+] (13), 201 (28), 171 (12), 143 (23), 128 (23), 115 (10), 110 (100), 91 (18), 59 (14).

***N*-(2-Methylpropyl)-6-phenyl-2(*E*),4(*E*)-hexadienamide) (5):** needles; mp 116 °C; UV λ_{max} (MeOH) 256 nm; IR ν_{max} (KBr disk) 3305, 2960, 2875, 1650, 1608, 1540, 1475 cm^{-1}; HREIMS 243.1566 ($C_{16}H_{21}NO$ requires 243.1623); EIMS *m/z* 243 [M^+] (75), 171 (54), 152 (32), 143 (28), 128 (91), 84 (100).

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References and Notes

(1) Horsham, M. A.; Class, T. J.; Johnston, J. J.; Casida, J. E. *J. Agric. Food Chem.* **1989**, *37*, 777–781.
(2) Johnston, J. J.; Horsham, M. A.; Class, T. J.; Casida, J. E. *J. Agric. Food Chem.* **1989**, *37*, 781–786.
(3) Elliot, M.; Farnham, A. W.; Janes, N. F.; Johnson, D. M.; Pulman, D. A. *Pestic. Sci.* **1987**, *18*, 191–201 (Part 1) to 239–244 (Part 6).
(4) Hartley, T. G. *Adansonia* **1997**, in press.
(5) Heal, R. E.; Rogers, E. F.; Wallace, R. T.; Starnes, O. *Lloydia* **1950**, *13*, 89–162.
(6) Jones, T. G. H.; White, M. *Proc. R. Soc. Queensland* **1929**, *41*, 154–157.
(7) The insect test species and their ages are as follows: two-spotted spider mite (*Tetranychus urticae*), adult; peach/potato aphid (*Myzus persicae*), mixed instars; housefly (*Musca domestica*), adult; tobacco budworm (*Heliothis virescens*), larvae; lesser armyworm (*Spodoptera exigua*), larvae; root knot nematode (*Meloidogyne incognita*), juveniles.
(8) Loder, J. W.; Moorhouse, A.; Russell, G. B. *Aust. J. Chem.* **1969**, *22*, 1531–1538.
(9) Okorie, D. A. *Phytochemistry* **1976**, *15*, 1799–1800.
(10) Su, H. C. F.; Horvat, R. *J. Agric. Food. Chem.* **1981**, *29*, 115–118.
(11) Kubo, I.; Matsumoto, T.; Klocke, J. A.; Kamikawa, T. *Experientia* **1984**, *40*, 340–341.
(12) Panichpol, K.; Waterman, P. G. *Phytochemistry* **1978**, *17*, 1363–1367.
(13) Murray, R. D. H.; Mendez, J.; Brown, S. A. *The Natural Coumarins, Occurrence, Chemistry and Biochemistry*; J. Wiley and Sons, Ltd.: Chichester, U.K., 1982.
(14) Latif, Z. Studies on the insecticidal components of tropical plants. Ph.D. Thesis, University of Strathclyde, 1997.
(15) Latif, Z.; Hartley, T. G.; Rice, M. J.; Waigh, R. D.; Waterman, P. G. *Biochem. Syst. Ecol.* **1997**, in press.
(16) Jacobson, M. In *Naturally Occurring Insecticides*; Jacobson, M., Crosby D. G., Eds.; Marcel Dekker: New York, 1971; pp 139–176.
(17) Greger, H. *Planta Med.* **1984**, 366–375.
(18) Elliot, M.; Farnham, A. W.; Janes, N. F.; Johnson, D. M.; Pulman, D. A.; Sawicki, R. M. *Agric. Biol. Chem.* **1986**, *50*, 1347–1349.
(19) Blade, R. In *Recent Advances in the Chemistry of Insect Control*; Crombie, L., Ed.; Royal Society of Chemistry: Cambridge, 1990; Vol. 2, pp 151–169.
(20) Crombie, L. *J. Chem. Soc.* **1955**, 999–1006.
(21) Berenbaum, M. *Science* **1978**, *201*, 532–534.
(22) Arnason, T.; Towers, G. H. N.; Philogene, B. J. R.; Lambert, J. D. H. In *Plant Resistance to Insects*; ACS Symposium Series No. 208; American Chemical Society: Washington, D.C., 1983; pp 139–151.
(23) Klocke, J. A.; Balandrin, M. F.; Barnby, M. A.; Bryan, R. In *Insecticides of Plant Origin*; ACS Symposium Series No. 387; American Chemical Society: Washington, 1989; pp136–149.
(24) Downum, K. R. *New Phytol.* **1992**, *122*, 401–420.
(25) Bowers, W. S. In *Phytochemical Resources for Medicine and Agriculture*; Nigg, H. N., Seigler, D., Eds.; Plenum Press: New York, 1992; pp 227–235.
(26) Miyakado, M.; Nakayama, I.; Ohno, N. In *Insecticides of Plant Origin*; ACS Symposium Series No. 387; American Chemical Society: Washington, 1989; pp 173–187.
(27) Potter, J. P. Zeneca Agrochemicals, U.K. Personal communication, 1996.
(28) Hartley, T. G. Personal communication, 1997.
(29) Hartley, T. G. *Gard. Bull. Singapore* **1981**, *34*, 91–131.
(30) Ng, K. M.; But, P. P. H.; Gray, A. I.; Hartley, T. G.; Kong, Y. C.; Waterman, P. G. *Biochem. Syst. Ecol.* **1987**, *15*, 587–593.
(31) Rice, M. J. Zeneca Agrochemicals, U.K., Personal communication, 1996.

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J.C.S. Faraday I, 1980, **76**, 2187–2193

Effect of the Nature of the Hydrophobic Group on the Mode of Association of Amphiphilic Molecules in Aqueous Solution

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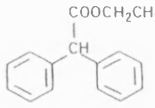
The importance of the flexibility of the hydrophobic group in determining the association pattern of an amphiphilic molecule has been examined by comparing the solution properties of a flexible diphenylmethane derivative, adiphenine hydrochloride, with those of a similar molecule, pavatrine hydrochloride, in which the two phenyl rings of the hydrophobic group are linked in the form of a rigid fluorene group. The concentration dependence of the light scattering from aqueous solutions of pavatrine hydrochloride showed no significant discontinuity attributable to a critical micelle concentration and could not be simulated using the mass-action model of micellisation, indicating that the association pattern does not conform to that of a monodisperse micellar system. In contrast, adiphenine hydrochloride exhibited a typical micellar association pattern.

The association of pavatrine hydrochloride in water may be represented by a two-parameter stepwise association model in which the dimerization constant is treated as an independent variable and higher association constants, K_N, decrease with increasing aggregation number, N, according to the relationship $K_N = K(N-1)/(N-2)$. Addition of sodium chloride causes not only an increase in the magnitude of the association constants but also a change in the pattern of association. In the presence of 0.1 mol dm^{-3} NaCl the association is best represented by a two-parameter model in which higher stepwise association constants are identical. At higher electrolyte concentrations (0.2 and 0.5 mol dm^{-3}) the association follows a one-parameter scheme in which association constants increase with N according to $K_N = K(N-1)/N$.

The mode of association of amphiphilic molecules is thought to be strongly influenced by the nature of the hydrophobic group.[1] In typical surfactants the hydrophobic group is a flexible hydrocarbon chain which can intertwine during the aggregation process to form approximately spheroidal micelles. In contrast, rigid planar molecules such as the cationic dyes [2] associate by face-to-face stacking in a stepwise association pattern. Whereas in micellar association there is a predominance of aggregates of an energetically preferred size, in stepwise association the products of association are generally multimers with a broad size distribution.

Previous studies [3] have established a micellar mode of association for amphiphilic drug molecules with diphenylmethane hydrophobic groups. Rotation around the central C atom of diphenylmethane clearly hinders stacking of these molecules. In this investigation a direct comparison is made of the association behaviour of the



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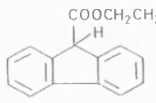

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diphenylmethane derivative, adiphenine hydrochloride, I, and that of a rigid analogue of this compound, pavatrine hydrochloride, II, in which the two phenyl rings of the hydrophobic group are linked in the form of a rigid fluorene group.

EXPERIMENTAL

MATERIALS

Pavatrine hydrochloride (2-diethylaminoethyl 9-fluorene carboxylate hydrochloride) was prepared by the interaction of 9-fluorenecarboxylic acid and β-diethylaminoethyl chloride.[4] The product was recrystallised from isopropanol and dried over phosphorus pentoxide under vacuum, m.p. 143°C [lit.[4] 143-144°C] i.r. and ^{1}H n.m.r. spectra were in accord with the structure and indicated a satisfactory degree of purity. (Found: C, 68.9; H, 7.2; N, 4.1. Calculated for $C_{20}H_{24}ClNO_2$: C, 69.45; H, 7.0; N, 4.05.) Sodium chloride was of AnalaR grade.

LIGHT SCATTERING MEASUREMENTS

A Fica 42000 photogoniodiffusometer (A.R.L. Ltd) was used at 303 K with incident light of wavelength 546 nm. Aqueous solutions were clarified by ultrafiltration through 0.1 μm Millipore filters until the ratio of light scattering at angles of 30 and 150° did not exceed 1.10. Refractive index increments determined at 546 nm using a differential refractometer were 0.070 kg mol^{-1} in water, 0.1 and 0.2 mol dm^{-3} NaCl and 0.067 kg mol^{-1} in 0.5 mol dm^{-3} NaCl.

RESULTS

The concentration dependence of the light scattering intensity at 90°, S_{90}, from solutions of pavatrine hydrochloride both in the presence and absence of added electrolyte is compared in fig. 1 with the previously reported [5] light scattering curve for adiphenine hydrochloride in water. The latter curve shows a marked deviation from the theoretical scattering line for unassociated monomers (calculated assuming thermodynamic ideality) at a well-defined critical micelle concentration (c.m.c.). In contrast the $S_{90}-m$ curves of pavatrine hydrochloride increase continuously with concentration, with no apparent inflection points. The absence of an inflection point does not itself constitute proof of a non-micellar type of association since the abruptness of the change in the scattering intensity at the c.m.c. is determined by the aggregation number, N, the micellar charge, p, and the equilibrium constant, K_m. A method of computer simulation of the light scattering curves based on the application of the law of mass action to the micellar process has been described previously.[6] The scattering intensity as a function of concentration was computed for a large series of combinations of the parameters K_m, N and the degree of dissociation, α ($= p/N$) in which N was varied in increments of 0.2 from 1.0 to 40.0 and α was varied in increments of 0.02 over the range 0.02-0.98. For each combination of N and α, K_m was adjusted by an iterative procedure to give the best fit to the experimental data as indicated by a goodness-of-fit parameter which was equally weighted over the entire concentration range. The light scattering results for adiphenine hydrochloride could be reproduced within the limits of error of the experimental data with $N = 10.0$, $K_m = 1.0 \times 10^{4.5}$ and $\alpha = 0.23$. It was not, however, possible to satisfactorily represent any of the $S_{90}-m$ curves of pavatrine hydrochloride using the mass action equation, indicating that the association pattern does not conform to that of a monodisperse micellar system.

The light-scattering data were analysed using curve-fitting procedures developed by Rosotti and Rosotti,[7] details of which have been previously given.[8] The variation

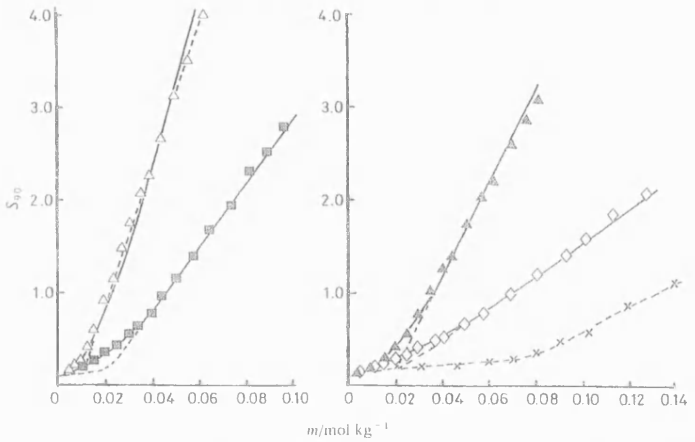

FIG. 1.—Concentration dependence of the scattering ratio, S_{90}, for pavatrine hydrochloride in ◇, H_2O; ▣, 0.1 mol dm^{-3} NaCl; ◬, 0.2 mol dm^{-3} NaCl; △, 0.5 mol dm^{-3} NaCl and ×, adiphenine hydrochloride in H_2O [from ref. (5)]. (— — —) Scattering intensity calculated using mass action equations for micellar association of pavatrine hydrochloride with $N = 7.6$, $K_m = 0.25 \times 10^{45}$, $\alpha = 0.10$ in water; $N = 17.4$, $K = 0.1 \times 10^{92}$, $\alpha = 0.20$ in 0.1 mol dm^{-3} NaCl; $N = 17.5$, $K = 0.13 \times 10^{97}$, $\alpha = 0.10$ in 0.2 mol dm^{-3} NaCl; and $N = 32.3$, $K = 0.32 \times 10^{178}$, $\alpha = 0.1$ in 0.5 mol dm^{-3} NaCl. For adiphenine hydrochloride in H_2O, $N = 10.0$, $K_m = 1.0 \times 10^{45}$ and $\alpha = 0.23$. (———) Theoretical scattering calculated using stepwise association models given in table 1.

of monomer concentration, b_1, with total solution concentration, m, was determined by integration of the light-scattering data according to

$$\ln x = \int_0^c [(M/M_{app}) - 1] \, d\ln c \tag{1}$$

x is the weight fraction of monomers, c is the weight concentration of the solution, M is the monomer molecular weight and M_{app} the apparent weight-average molecular weight calculated from the light-scattering intensity assuming ideality.

Several models of self-association were considered, the number of variables being restricted to one or two. In one-parameter models an explicit relationship between all stepwise association constants was assumed, the relationship being expressed using a generalized parameter K. In two-parameter models the association constant for dimerization, K_2 was considered as an independent variable and higher K_N values related to K.

The one-parameter models considered were [2]

Model X: In this, the simplest one-parameter model, the equality of all K values is assumed leading to the relation

$$(b_1/m)^{\frac{1}{2}} = 1 - Kb_1. \tag{2}$$

Eqn (2) gave poor representation of the experimental data in all systems.

Model Y: Stepwise association constants increase sequentially with N according to

$$K_N = K(N-1)/N \tag{3}$$

leading to the relation

$$m/b_1 = 1 + Km. \quad (4)$$

Fig. 2 shows linear relationship between m/b_1 and m for pavatrine hydrochloride in 0.2 and 0.5 mol dm^{-3} NaCl. Values of K from the slope of the plots for these two systems are given in table 1. In the remaining systems, model Y gave unsatisfactory fits of the experimental data and these systems were analysed using two-parameter models.

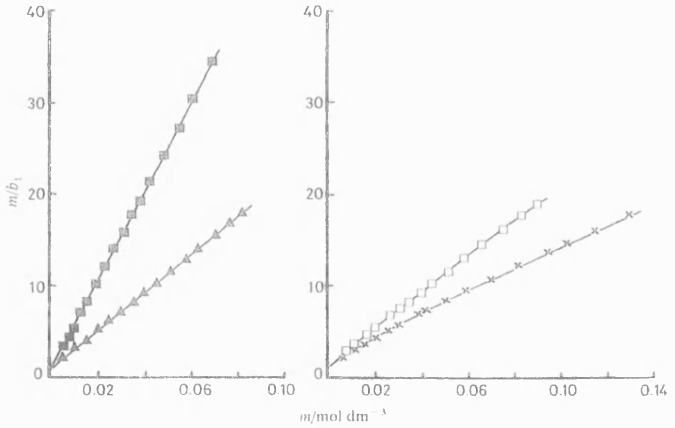

FIG. 2.—Data plotted according to one-parameter association model Y for pavatrine hydrochloride in ×, H_2O; □, 0.1 mol dm^{-3} NaCl; ▲, 0.2 mol dm^{-3} NaCl; ▩, 0.5 mol dm^{-3} NaCl.

TABLE 1.—EFFECT OF ELECTROLYTE ON THE PATTERN OF ASSOCIATION OF PAVATRINE HYDROCHLORIDE IN AQUEOUS SOLUTION

[NaCl] /mol dm^{-3}	association model		association constants /dm^3 mol^{-1} K_2	K
0.00	$K_N = K(N-1)/(N-2)$	$N \geqslant 3$	95	76
0.10	$K_N = K$	$N \geqslant 3$	98	180
0.20	$K_N = K(N-1)/N$	$N \geqslant 2$	101	202
0.50			238	476

In two-parameter association models, plots of log T against log b were compared with normalized curves of log T^* against log b^* for each association model, where

$$b^* = K b_1 \quad (5)$$

$$T^* = TK/K_2 \quad (6)$$

$$T = (m - b_1)b_1. \quad (7)$$

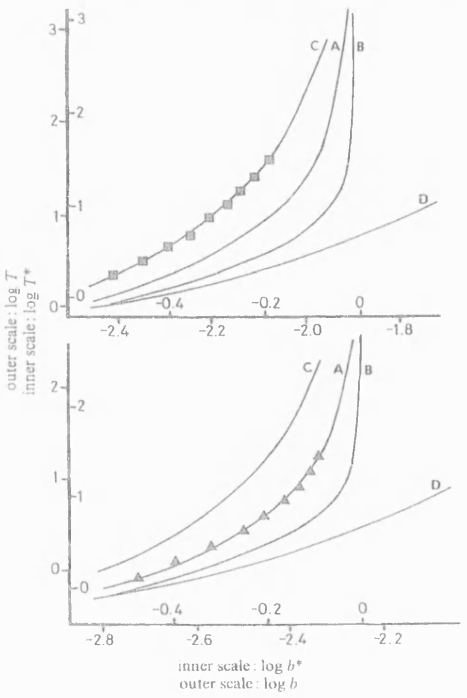

FIG. 3.—Experimental log T against log b data for pavatrine hydrochloride in ▩, water and ▲, 0.1 mol dm^{-3} NaCl superimposed on normalized curves of log T^* against log b^* for two-parameter association models described by eqn (8)-(15).

The four models considered are defined in eqn (8)-(15).

Model A : Stepwise association constants are identical for $N \geqslant 3$

i.e.

$$K_3 = K_4 = K_N = K \tag{8}$$

$$\text{and } T^* = b^*(2-b^*)/(1-b^*)^2 \text{ for } b^* < 1. \tag{9}$$

Model B : Stepwise association constants increase in a mild sequence according to

$$K_N = K(N-2)/(N-1) \text{ for } N \geqslant 3 \tag{10}$$

$$\text{and } T^* = [b^*/(1-b^*)] - \ln(1-b^*) \text{ for } b^* < 1. \tag{11}$$

Model C : Stepwise association constants decrease in a mild sequence according to

$$K_N = K(N-1)/(N-2) \text{ for } N \geqslant 3 \tag{12}$$

$$\text{and } T^* = 2b^*/(1-b^*)^3 \text{ for } b^* < 1. \tag{13}$$

Model D: Stepwise association constants decrease relatively rapidly with N according to

$$K_N = K/(N-1) \text{ for } N \geqslant 3 \tag{14}$$

$$\text{and} \quad T^* = e^{b^*}(1+b^*) - 1 \text{ for } b^* < 1. \tag{15}$$

The experimental log T against log b_1 curves for pavatrine hydrochloride in water and in the presence of 0.1 mol dm^{-3} electrolyte are compared with the normalized curves in fig. 3. This figure shows clearly the change in the pattern of stepwise association from a model in which K_N decreases with increasing N in water to one in which K_N increases with N in the presence of electrolyte. K_2 and K values were calculated from the displacements of the coordinate axes required to achieve the best fit of the curves, using eqn (5) and (6).

Values of the association constants derived from the one- and two-parameter schemes were used to recalculate the light scattering curves in the following way.

The weight-average degree of association, N_w, of the system including the monomers is given by

$$N_w = G/C \tag{16}$$

where $C = \sum_2^N N[b_N]$ and $G = \sum_2^N N^2[b_N]$.

For an associating system conforming to the one-parameter model Y, summation of the series for G and C yields

$$C = b_1/(1-X) \tag{17}$$

$$G = b_1/(1-X)^2 \tag{18}$$

where $X = Kb_1$.

The weight-average degree of association is thus

$$N_w = 1/1-X. \tag{19}$$

A similar treatment for the two-parameter model A yields

$$C = \frac{X(K-K_2)}{K^2} + \frac{K_2X}{K^2}\left[\frac{1}{(1-X)^2}\right] \tag{20}$$

$$G = \frac{X(K-K_2)}{K^2} + \frac{K_2X}{K^2}\left[\frac{1+X}{(1-X)^3}\right]. \tag{21}$$

Hence

$$N_w = \frac{X(K-K_2)(1-X)^3 + K_2X(1+X)}{X(K-K_2)(1-X)^3 + K_2X(1-X)}. \tag{22}$$

Similarly, for the two-parameter model C

$$C = b_1 + \frac{2K_2X^2}{K^2}\left[\frac{1}{(1-X)^3}\right] \tag{23}$$

$$G = b_1 + \frac{2K_2X^2}{K^2}\left[\frac{2+X}{(1-X)^4}\right]. \tag{24}$$

Hence

$$N_w = \frac{XK(1-X)^4 + 2K_2X^2(2+X)}{XK(1-X)^4 + 2K_2X^2(1-X)} \quad (25)$$

The scattering intensity was calculated as a function of concentration from the values of N_w for the appropriate association model, assuming ideality. Eqn (19), (22) and (25) were used where necessary to improve the initial estimates of K by an iterative method. Fig. 1 shows a satisfactory fit of the experimental data for all systems using the association models and constants given in table 1.

DISCUSSION

It has been shown in this investigation that replacement of a flexible, diphenylmethane group of an amphiphilic molecule with a rigid, planar fluorene group can result in conversion from a micellar to a non-micellar mode of association. Note however that, although rigidity of the hydrophobic group is clearly an important requirement for non-micellar association, it is not the only requirement. A large number of amphiphilic molecules with rigid tricyclic hydrophobic groups, such as the phenothiazines [9] and antidepressant drugs,[10] associate in a micellar manner. Other factors affecting the mode of association will be reported in later papers.

In an investigation of the effect of ionic strength on the association of the cationic dye methylene blue, Ghosh and Mukerjee [2] noted only a small counterion effect on the magnitude of the association constants. Similar conclusions were reported by Padday [11] for the association of other dyes. Although pavatrine hydrochloride, like methylene blue, possesses a planar, tricyclic ring system it bears a closer resemblance to conventional surfactants since its charge is localised at the end of the side chain rather than delocalised as in methylene blue. In typical surfactants, electrolyte addition leads to an increase in aggregation number and a lowering of the c.m.c. due to a reduction in the repulsive interaction of the ionic head groups. In this investigation it is shown that the reduction of this repulsive interaction not only causes an increase in the mean aggregate size but also profoundly affects the pattern of association; converting from a model in water showing anticooperativity to one in the presence of electrolyte which exhibits cooperativity.

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[1] P. Mukerjee, *J. Pharm. Sci.*, 1974, **63**, 972.
[2] A. K. Ghosh and P. Mukerjee, *J. Amer. Chem. Soc.*, 1970, **92**, 6408, 6413.
[3] D. Attwood, in *Techniques and Applications of Fast Reactions in Solution*, ed. W. J. Gettins and E. Wyn-Jones (D. Reidel, Dordrecht, 1979), p. 201.
[4] R. R. Burtner and J. W. Cusic, *J. Amer. Chem. Soc.*, 1943, **65**, 262.
[5] D. Attwood, *J. Pharm. Pharmacol.*, 1976, **28**, 407.
[6] D. Attwood and O. K. Udeala, *J. Phys. Chem.*, 1975, **79**, 889.
[7] F. J. C. Rosotti and H. Rosotti, *J. Phys. Chem.*, 1961, **65**, 926, 930.
[8] D. Attwood and S. P. Agarwal, *J.C.S. Faraday I*, 1980, **76**, 570.
[9] D. Attwood, A. T. Florence and J. M. N. Gillan, *J. Pharm. Sci.*, 1974, **63**, 988.
[10] D. Attwood and J. Gibson, *J. Pharm. Pharmacol.*, 1978, **30**, 176.
[11] J. F. Padday, *J. Phys. Chem.*, 1967, **71**, 3488; 1968, **72**, 1259.

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REFERENCES

Ali, B., Gupta, K. P., Kumar, A., Bhargava, K. P. (1980) Pharmacology 20: 181–187

Ballinger, B., Browning, M., O'Malley, K., Stevenson, I. H. (1972) Br. J. Pharmacol. 45: 638–643

Brown, S. S., Goenechea, S. (1973) Clin. Pharmacol. Ther. 14: 314–324

Drach, J. C., Howell, J. P., Borondy, P. E., Glazko, A. J. (1970) Proc. Soc. Exp. Biol. Med. 135: 849–853

Gujral, M. L., Sareen, K. N., Kohli, R. P. (1957) Indian J. Med. Res. 45: 207–212

Gujral, M. L., Saxena, P. N., Tewari, R. S. (1955) Ibid. 43: 637–641

Hudmarsh, K. W., Hamon, N. W., Le Gatt, D. F., Wallace, S. M. (1978) J. Pharm. Sci. 67: 1547–1550

Kappas, A., Anderson, K. E., Conney, A. H., Alvares, A. P. (1976) Clin. Pharmacol. Ther. 20: 643–653

Norris, W., Telfer, A. B. M. (1969) Br. J. Anaesth. 41: 874–876

Notari, R. E., De Young, J. L., Anderson, K. E. (1975) Biopharmaceutics and Pharmacokinetics. An Introduction. Second Edition. Marcel Dekker Inc. New York.

Nowak, H., Schorre, G., Struller, R. (1966) Arzneim. Forsch. 16: 407–411

Parmar, S. S., Ali, B., Brumleve, S. J., De Boer, B. (1974) Pharmacologist 16: 238

Pascarelli, E. F. (1973) J. Am. Med. Assoc. 224: 1512–1514

Prabhu, V. G. Browne, R. K., Zaroslinski, J. F. (1964) Int. Pharmacodyn. Ther. 148: 228–236

Prabhu, V. G., Parikh, S. H., Shah, G. F. (1967) Indian J. Med. Sci. 21: 524–532

Preuss, Fr. R., Hassler, H. M., Köpf, R. (1966) Arzneim.-Forsch. 16: 401–407

Seth, P. K., Srivastava, S. P., Agarwal, D. K., Mushtaq, M. (1977) Bull. Environ. Contam. Toxicol. 17: 727–732

Temant, S. F. Jr. (1973) Br. J. Addict. 68: 327–330

J. Pharm. Pharmacol. 1982, 34: 746–749
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Parabolic structure-activity relationships: a simple pharmacokinetic model

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Several models have been proposed for the parabolic relationship that many drugs show between pharmacological response and lipophilicity. Hansch and coworkers (Penniston et al 1969; Hansch & Clayton 1973) have proposed that the parabolic relationship arises from the passive diffusion of the drug through alternating aqueous and lipid phases and produced computer simulations to substantiate this argument. McFarland (1970) also considered a system comprising alternating aqueous and lipid phases and using probability arguments derived a bilinear equation to describe the relationship between pharmacological response and lipophilicity. Kubinyi (1976, 1977) has extended McFarland's work and reported that the bilinear model explains most of the data in the literature better than Hansch's quadratic model.

Since an in vivo biological system is much more complicated than a series of alternating aqueous and lipid phases, these models must be viewed as empirical rather than fundamental. Consequently we will use the term 'parabolic' to describe the situation in which, amongst a group of compounds with varying lipophilicity one compound elicits the largest pharmacological response (per unit dose). The term is not meant to imply quadratic in the sense of Hansch.

All of the approaches that have been proposed so far have as their basis the postulate that pharmacological response is determined by the ability of the drug to reach its receptor site. While this postulate is undoubtedly correct, the contribution of the drug's pharmacokinetics to its concentration at the receptor site has been neglected. Thus all of the proposed models are closed in that the drug accumulates at the receptor site. It was the purpose of the present study to investigate, in the most elementary fashion, the impact of pharmacokinetics on structure-activity relationships.

* Correspondence.

Closed model

The simplest example of alternating aqueous and lipid phases consists of an aqueous-lipid-aqueous sequence as shown in Fig. 1. The aqueous to lipid rate constant is k_1 and the lipid to aqueous rate constant is k_2. Assuming that the volumes of the three compartments are equal, the rate equations governing the drug concentration in the three compartments are

$$\frac{dC_1}{dt} = -k_1C_1 + k_2C_2 \quad (1)$$

$$\frac{dC_2}{dt} = k_1C_1 - 2k_2C_2 + k_1C_3$$

$$\frac{dC_3}{dt} = k_2C_2 - k_1C_3$$

Introducing a reduced time, $\tau = k_2 t$, and dimensionless concentrations, $C_1' = C_1/C_1(0)$, $C_2' = C_2/C_1(0)$ and $C_3' = C_3/C_1(0)$ where $C_1(0)$ is the concentration of drug in compartment 1 at time zero, equations (1) become

$$\frac{dC_1'}{d\tau} = -PC_1' + C_2' \quad (2)$$

$$\frac{dC_2'}{d\tau} = PC_1' - 2C_2' + PC_3'$$

$$\frac{dC_3'}{d\tau} = C_2' - PC_3'$$

where $P = k_1/k_2$ represents the drug's lipophilicity. If the drug is introduced as a bolus into compartment 1 the solution for C_3', the proposed receptor site, is

$$C_3'(\tau) = \frac{[2 + Pe^{-(P+2)\tau} - (P+2)e^{-P\tau}]}{2(P+2)} \quad (3)$$

This equation has been extensively studied by Cooper et al (1981) and they found that for any fixed value of τ there is a value of P for which $C_3'(\tau)$ is maximal. The results of these studies are summarized in Fig. 2 which shows the relationship between optimal lipophilicity, P_{opt}, and sampling time, τ. It can be seen that as the sampling time becomes longer the optimal lipophilicity decreases. When the variables are transformed back into their original units it becomes apparent that for each combination of τ and P_{opt} there is an infinite set of values for k_1, k_2 and t which satisfy the relationships $P_{opt} = k_1/k_2$ and $\tau = k_2 t$.

There are two problems associated with this model. Firstly the sampling time is arbitrary and consequently so is the optimal lipophilicity. Secondly, the model is closed in that the ultimate concentration in compartment 3 decreases monotonically as a function of P, viz

$$C_3'(\infty) = \frac{1}{P+2} \quad (4)$$

If the drug equilibrates with the receptor site in a time much shorter than that required for distribution equilibrium with other tissues and for drug elimination, then the closed model, under the pseudo steady-state hypothesis, is a good representation of the in vivo situation: otherwise it is not.

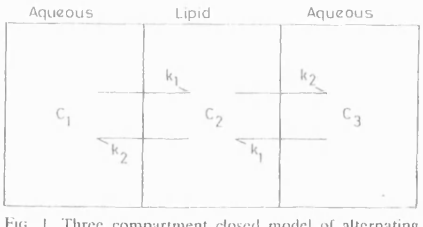

FIG. 1. Three compartment closed model of alternating aqueous-lipid phases.

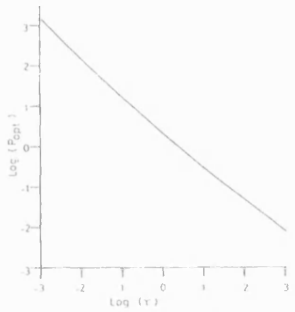

FIG. 2. Optimal lipophilicity, P_{opt}, as a function of sampling time, τ, for a three compartment closed model.

Open model

The previous model can be made open by the inclusion of an exit rate constant, k, from compartment 1 as shown in Fig. 3. Although the model is not mammalian in the sense of usual pharmacokinetic models, it is the simplest open model which shows parabolic behaviour. The equations for this model are

$$\frac{dC_1'}{d\tau} = PC_1' + C_2' - k'C_1' \quad (5)$$

$$\frac{dC_2'}{d\tau} = PC_1' - 2C_2' + PC_3'$$

$$\frac{dC_3'}{d\tau} = C_2' - PC_3'$$

where $k' = k/k_2$. The solution for C_3' is

$$C_3' = P\left[\frac{e^{\lambda_1\tau}}{(\lambda_1-\lambda_2)(\lambda_1-\lambda_3)} + \frac{e^{\lambda_2\tau}}{(\lambda_2-\lambda_1)(\lambda_2-\lambda_3)} + \frac{e^{\lambda_3\tau}}{(\lambda_3-\lambda_1)(\lambda_3-\lambda_2)}\right] \quad (6)$$

Where λ_i are the roots of the cubic equation

$$\lambda^3 + (2P + k' + 2)\lambda^2 + (P^2 + 2P + k'P + 2k')\lambda + k'P = 0 \quad (7)$$

Simulations using equation (6) for $k' = 1$ and various values of P demonstrate that there is an optimal value for P for which the peak concentration is maximal (see Fig. 4). If the peak concentration is equated to peak response this means that there is a parabolic relationship between response and lipophilicity.

Further simulations confirmed this finding. The effect of lipophilicity on peak concentration in compartment

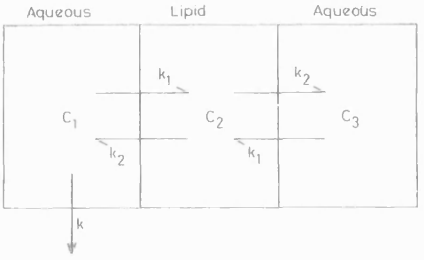

FIG. 3. Three compartment open model.

3, for several values of k′, is shown in Fig. 5. The bilinear nature of the curves is evident. It can be seen that as k increases the optimal lipophilicity also increases at the expense of the peak concentration, which decreases. This behaviour is summarized in Fig. 6. As with the closed model there is an infinite set of values of the variables k_1, k_2 and k, satisfying the relationships $P_{opt} = k_1/k_2$ and $k' = k/k_2$, corresponding to any given combination of k′ and P_{opt}.

Discussion

Two variations of the open model were studied. Firstly elimination from compartment 3 was considered and secondly a restraint was placed on the rate constants, k_1 and k_2. There is some evidence (Lippold & Schneider 1975; Van de Waterbeemd et al 1980) that, far from increasing to infinity, these rate constants reach limiting values. One possible explanation is that, when the rate of transfer between the two phases becomes so rapid, the rate limiting step becomes not the transfer, but diffusion to the interface. Consequently we tried imposing the following restrictions on the rate constants (similar constraints were used by Van de Waterbeemd et al 1980)

$$k_1 = \frac{a\,P}{b+P} \qquad (8)$$

$$k_2 = \frac{a}{b+P}$$

where a and b are constants. These two variations on the basic model produced quantitative changes but the parabolic behaviour was still retained. Unlike previously, when the constraints of equation (8) are applied, to each combination of k′ and P_{opt} there corresponds only one set of values for the variables k_1, k_2 and k.

It was not the purpose of the present study to produce

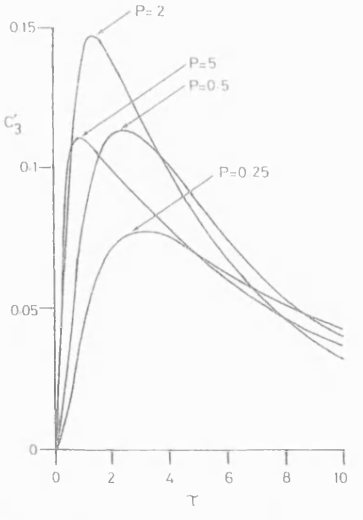

FIG. 4. Effect of lipophilicity, P, on the concentration (C_3)-time (τ) profile in compartment 3 of a three-compartment open model. The elimination rate constant, k′, is equal to 1.

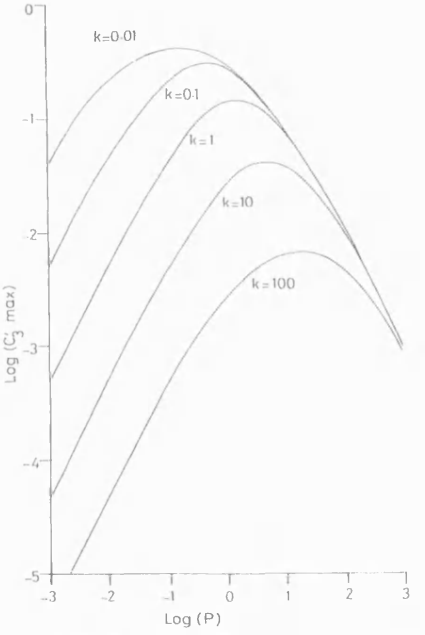

FIG. 5. Effect of lipophilicity, P, on peak concentration in compartment 3 (C_3' max) of a three-compartment open model as a function of elimination rate constant, k′.

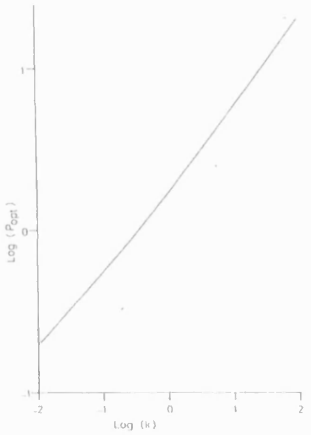

FIG. 6. Optimal lipophilicity, P_{opt}, as a function of elimination rate constant, k', for a three-compartment open model.

a definitive model which would relate molecular structure to pharmacological activity. Instead we raise several issues which, we feel, have been treated inadequately. The validity of the model herein described and that of previous models is not established by the fact that they predict parabolic structure-activity relationships. In order to define a structurally valid in vivo model detailed pharmacokinetic experiments are needed: preferably on a homologous group of compounds. From the results of these experiments it would be possible to define the effect of structure, perhaps in the guise of a parameter such as lipophilicity, on fundamental pharmacokinetic parameters such as clearance and volume of distribution. However it would then be necessary to separate the effect of structure on a drug's pharmacokinetics and its pharmacological response. Pharmacological activity is related to the drug concentration at the receptor site whereas most pharmacokinetic studies are restricted to measurements of drug concentration in blood or plasma. Consequently it is often difficult to relate the pharmacokinetics of a drug to its pharmacodynamics. In the simplest situation pharmacological response is directly related to blood or plasma concentration. However, if the receptor site is in a tissue which does not rapidly equilibrate with blood it may not be possible to separate structural effects associated with pharmacokinetics from those associated with pharmacological response.

At the present time detailed experiments such as those described above do not exist. Consequently we have not elaborated on the simple model discussed in this paper but we believe it to be a more realistic representation of the in vivo situation than previously described closed models.

Since the paper was submitted a comprehensive review of the existing literature on structure-pharmacokinetic relationships has appeared (Seydel & Schaper 1982).

REFERENCES

Cooper, E. R., Berner, B., Bruce, R. (1981) J. Pharm. Sci. 70: 57–59

Hansch, C., Clayton, J. M. (1973) Ibid. 62: 1–21

Kubinyi, H. (1976) Arzneim.-Forsch. 26: 1991–1997

Kubinyi, H. (1977) J. Med. Chem. 20: 625–629

Lippold, V. B. C., Schneider, G. F. (1975) Arzneim.-Forsch. 25: 843–852

McFarland, J. W. (1970) J. Med. Chem. 13: 1192–1196

Penniston, J. T., Beckett, L., Bentley, D. L., Hansch, C. (1969) Mol. Pharmacol. 5: 333–341

Seydel, J. K., Schaper, K.-J. (1982) Pharmac. Ther. 18: 131–182

Van de Waterbeemd, H., Van Boeckel, S., Jansen, A., Gerritsma, K. (1980) Eur. J. Med. Chem. 15: 279–282

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Some Observations on the WATR Method for Water Suppression at 80 MHz

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The WATR method for water suppression in ^{1}H NMR spectroscopy has been evaluated at 80 MHz, using both ammonium and guanidinium chloride, with and without the addition of buffer salts. The pH of maximum effect has been found to depend on field strength and to be markedly affected by buffer salts, particularly phosphates. Provided that the pH is carefully controlled the method is simple and effective.

KEY WORDS ^{1}H NMR WATR Water suppression Ammonium chloride Guanidinium chloride Buffer salts

It has been shown that effective water suppression in proton NMR spectra can be achieved using ammonium chloride as a relaxation enhancer.[1] Chemical exchange between water and the added reagent results in considerable shortening of the spin–spin relaxation time of the exchanging protons: a Carr–Purcell–Meiboom–Gill spin–echo sequence then selectively removes the water signal, provided that the cumulative delay is sufficient.

Our results with ammonium chloride confirm that the effect is highly pH dependent. However, our experiments at 80 MHz suggest that the greatest effect occurs at *ca* pH 5 in unbuffered solutions, unlike the results of the original authors at 360 MHz, where the greatest effect was observed at *ca* pH 6.5.[1]

We have also tested the hypothesis that other selected nitrogenous compounds will show effects similar to those of ammonium chloride. Of the relatively simple analogues, guanidine salts appeared to offer two possible advantages, since on a molar basis the guanidinium ion has two more exchangeable protons than the ammonium ion and a much higher pK_a value[2] of 14.46 in water at 25 °C compared with 9.3 for ammonium. Guanidinium might therefore be expected to be more potent on a molar basis and possibly to have a different pH of maximum effect. We have also examined the effects of urea, which is present naturally in urine samples. When this work was nearing completion, a detailed account of the use of guanidinium chloride and hydroxylamine hydrochloride appeared.[3] In this report we have therefore concentrated on those aspects of our work which complement the results of the other authors.

As is shown in Fig. 1, guanidinium chloride was slightly more effective at 80 MHz than ammonium chloride on a molar basis. There was a marked difference in the pH of maximum effect, however, that for guanidinium occurring at *ca* pH 7 when an 80 MHz spectrometer was used. Again, there is evidence for field dependence of the pH for maximum effect from some results obtained for comparison at 500 MHz, where the maximum effect was obtained at *ca* pH 8 (Fig. 2).

One further difference between the two reagents emerged when buffer solutions were used to stabilize the pH for ammonium chloride solutions. Unlike guanidinium chloride solutions, different buffers with ammonium chloride produced discontinuities in the plot of log T_2 against pH when the buffers were used in their normal ranges (phthalate buffer in the pH range 4.2–6.2, phosphate at pH 5.0–8.0 and borate at pH 7.8–9.2). Use of

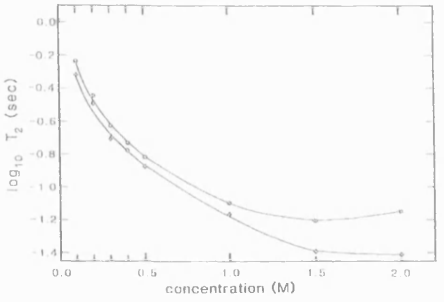

Figure 1. Concentration dependence of log T_2 at 80 MHz on (●) ammonium chloride and (◈) guanidinium chloride.

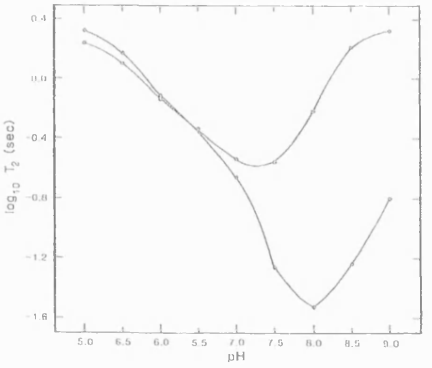

Figure 2. pH dependence of log T_2 for water in the presence of guanidinium chloride (0.25 M). pH 5.0–5.5, phthalate buffer; pH 6.0–7.5, phosphate buffer; pH 8.0–9.0, borate buffer. (●), 80 MHz; (◈), 500 MHz.

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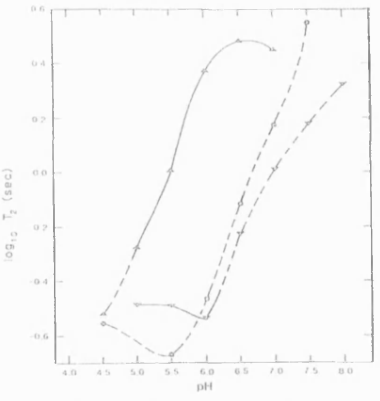

Figure 3. pH dependence of log T_2 at 80 MHz for water in the presence of ammonium chloride (0.25 M), (△), Phosphate buffer; (▽), phthalate buffer; (⊘), borate buffer. Dashed lines represent buffer salts outside useful buffering range.

buffer solutions beyond their normal ranges showed that the apparent discontinuities were caused by differences in the pH of maximum effect (Fig. 3).

Various observations may be made concerning the use of guanidine hydrochloride to suppress the water signal. Guanidine is such a strong base that the natural pH of solutions of its salts is close to pH 7, and little adjustment of most solutions is required to reach the pH of maximum effect at 80 MHz. Since guanidine is for practical purposes completely protonated at pH 7, the observed relaxation effect must occur with the guanidinium cation. This is the dominant species over the whole of the aqueous pH region, so the pH dependence of the effect must arise from interactions between the water and the solute rather than from the solute alone.

The use of mixtures of reagents has been recommended[3] in order to widen the pH range. We can confirm that this is effective (Fig. 4) but note that good water suppression was achieved without buffer over the range pH 5–7; at higher field the reported effective range[3] for a mixture of these two salts was pH 6–9.

An example of the use of guanidine hydrochloride to suppress the water signal at 80 MHz is given in the spectrum of procaine hydrochloride (Fig. 5). The methylene protons adjacent to the ester oxygen can be clearly seen, even though they lie very close to the water resonance frequency. The method is particularly useful at low field owing to the problems encountered with other methods of water suppression, despite the relatively small reduction in T_2 compared with high field (Fig. 2).

Urea proved to be less effective as a relaxation enhancer than either ammonium or guanidinium chloride and had little effect on relaxation produced by the other reagents. It may be preferable to hydroxylamine for water suppression at low pH values since urea is not toxic.

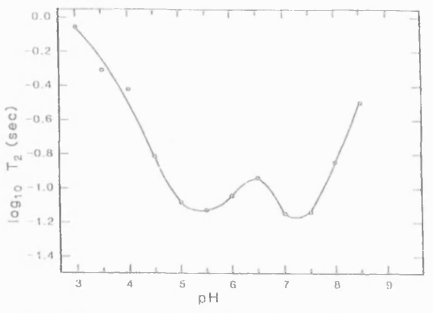

Figure 4. pH dependence of log T_2 at 80 MHz with a mixture of ammonium and guanidinium chloride (each 0.25 M) in unbuffered solution.

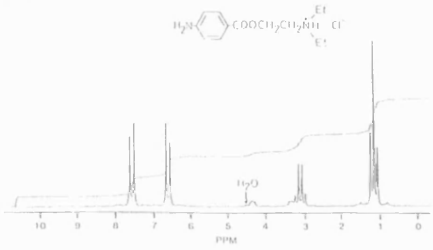

Figure 5. ^{1}H 80 MHz spin–echo spectrum of procaine hydrochloride (0.2 M) in 0.1 M phosphate buffer adjusted to pH 7 and containing 1.0 M guanidinium chloride. Total delay in Carr–Purcell–Meiboom–Gill sequence, 1.2 s.

EXPERIMENTAL

The experiments were carried out on a Bruker WP80 NMR spectrometer at 35 °C, except for those at 500 MHz, which were obtained on a Bruker AM 500 instrument at 25 °C. Solutions made with distilled water were adjusted to the required pH before adding sufficient deuterium oxide for an internal lock and adjusted to volume. The CPMG pulse sequence employed echoes of 1 ms duration. A spectral width of 1500 Hz was used throughout. Digital data printouts were used to find T_2 by least-squares regression analysis. The magnitude of T_2 is affected by other factors such as the oxygen concentration; solutions made with boiled distilled water gave longer relaxation times but did not differ in the pH of maximum effect.

Acknowledgements

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REFERENCES

1. D. L. Rabenstein, S. Fan and T. T. Nakashima, *J. Magn. Reson.* 64, 541 (1985).
2. M. Charton, *J. Org. Chem.* 30, 969 (1965).
3. D. L. Rabenstein and S. Fan, *Anal. Chem.* 58, 3178 (1986).

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0066-4804/88/111693-06$02.00/0

Antibacterial Action of 2-Bromo-2-Nitropropane-1,3-Diol (Bronopol)

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Patterns of growth inhibition of *Escherichia coli* in the presence of 2-bromo-2-nitropropane-1,3-diol (bronopol) indicate a period of biocide-induced bacteriostasis followed by growth at an inhibited rate. The length of the bacteriostatic period, but not the subsequent growth inhibition, was reduced by the addition of excess cysteine. Patterns of growth inhibition were unaffected by catalase or superoxide dismutase. The bactericidal concentrations (100 to 500 μg/ml) were considerably in excess of the MIC (13 μg/ml) and generally produced first-order reductions in viability. Bactericidal activity was considerably reduced by anoxic conditions and by the presence of catalase or superoxide dismutase. Results indicate that there are two distinct reactions between bronopol and thiols. Under aerobic conditions, bronopol catalytically oxidizes thiol-containing materials such as cysteine, with atmospheric oxygen as the final oxidant. By-products of this reaction are active oxygen species such as superoxide and peroxide, which are directly responsible for the bactericidal activity of the compound and for the reduced growth rate after the bacteriostatic period. The latter effect probably results from the oxidation of intracellular thiols such as glutathione and cysteine. Catalytic oxidation of thiols in the presence of excess thiol leads to the creation of an anoxic state. Under these conditions, the slower reaction with thiols, which consumes bronopol, predominates. Consumption of bronopol by its reaction with thiols, without the involvement of oxygen, leads to the eventual removal of bronopol from treated suspensions and the resumption of growth.

2-Bromo-2-nitropropane-1,3-diol (bronopol) has a broad spectrum of antibacterial activity (12) and is widely used, at concentrations of up to 0.1% (wt/vol), as a preservative for pharmaceutical and cosmetic products (4, 15). Previous studies of the mechanism of action of bronopol all conclude that the antibacterial activity of bronopol relates to its interaction with essential thiols within the cell (3, 16, 18). Such interaction is thought to lead to the oxidation of thiols through a radical anion intermediate (11). Unlike other thiol-interactive antimicrobial agents, bronopol possesses significant bactericidal activity that cannot be explained solely in terms of thiol oxidation.

This paper examines the hypothesis that separate actions are responsible for the growth inhibitory and bactericidal activities of the compound.

MATERIALS AND METHODS

Organisms, chemicals, and culture maintenance. *Escherichia coli* ATCC 8739 was used throughout the study. Cultures were maintained on nutrient agar (Oxoid CM 3) slopes at room temperature in the dark after incubation at 35°C. 2-Bromo-2-nitropropane-1,3-diol (bronopol) was obtained from the Boots Chemical Co., Nottingham, England. Catalase, superoxide dismutase, cytochrome *c*, cysteine, cystine, cysteine hydrochloride, and cystine dimethyl ester were obtained from the Sigma Chemical Co., St. Louis, Mo. All other reagents were of the purest available grade obtainable from BDH, Poole, England.

Growth inhibitory activity. To a series of Erlenmeyer flasks (250 ml) containing 98 ml of chemically defined simple salt medium (1) were added 1-ml portions of a similarly grown overnight culture. The flasks were shaken in an orbital incubator (35°C, 200 oscillations per min), and optical density measurements at 470 nm ($E_{470\ nm}$) were made at 15-min intervals. When the cultures were in the logarithmic phase of growth and $E_{470\ nm}$ was 0.15, various concentrations of a bronopol solution were added (1 ml). Growth was monitored in the flasks by optical density measurements for a further 2 h. In selected experiments, cysteine was added at various times following the addition of biocide to give molar ratios of cysteine to biocide of 10:1 and 1:1. In other experiments, catalase (50 U/ml) or superoxide dismutase (60 U/ml) was added simultaneously with bronopol. Experiments were performed in duplicate.

Preparation of washed cell suspensions. Erlenmeyer flasks (250 ml) containing 100 ml of a chemically defined simple salt medium (1) were inoculated from nutrient agar slopes and incubated at 35°C in an orbital incubator (200 oscillations per min) for 16 h. The cells were harvested by centrifugation (10,000 × *g*, 15 min, 35°C), washed twice in sterile saline (0.9%, wt/vol), and suspended to an appropriate optical density in phosphate-buffered saline (pH 7.0, 0.1 M). Suspensions were used within 1 h of preparation.

Bactericidal activity. Washed suspensions of *E. coli* (19 ml, 5×10^8 cells per ml) in Erlenmeyer flasks (100 ml) were equilibrated at 35°C in an orbital incubator (200 oscillations per min) for 30 min prior to the addition of biocide (1 ml). At appropriate times, 1-ml portions were removed and serially diluted in thioglycolate medium (Oxoid CM173). Suitable dilutions (0.1 ml) were spread on the surfaces of triplicate predried (50°C, 30 min) nutrient agar plates. Viable counts were determined following incubation of the plates at 35°C for 16 h. Preliminary experiments confirmed that dilution (1:10) of bronopol, at the concentrations used, in thioglycolate medium completely neutralized the bactericidal effect. Experiments were repeated at various temperatures at pH 7.0 and at various pH values at 35°C. Selected experiments were performed in the presence of catalase (100 U/ml) or superoxide dismutase (200 U/ml) or under anoxic conditions obtained by flushing the culture media with nitrogen before inoculation and performing the experiment in static tubes sealed from the atmosphere by a layer of light liquid paraffin.

Chemical analyses. Chemical analyses of the reaction

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products of bronopol-thiol mixtures were conducted by performing the reactions in 5-mm-diameter nuclear magnetic resonance (NMR) tubes and obtaining ^{1}H and ^{13}C spectra by using a Fourier transform NMR spectrometer at 80 MHz (Bruker WP80 SY). Proton and two-dimensional ^{1}H-^{13}C (HETCOR) spectra of isolated reaction products were obtained by using a 300-MHz, Fourier transform spectrometer (Varian XL-300). Infrared and mass spectroscopy were conducted with a Pye Unicam SP 3-300 spectrometer and Kratos MS 25 spectrometer, respectively.

Thin-layer chromatographic (TLC) analysis of reaction products was conducted on Kiesel gel 60 TLC plates (Macherey-Nagel, Duren) with butan-1-ol-glacial acetic acid-water (60:15:25). Plates were developed either with ninhydrin in acetone (0.2%, wt/vol) (13) or with sodium carbonate solution (10%, wt/vol), followed by 0.7 N Folin-Ciocalteau reagent (14). The latter stain was superior for visualization of the bronopol spots.

Gas chromatographic (GC) analysis of reaction products was performed with a Hewlett-Packard 5840A gas chromatograph, 7671A automatic sampler, and 5840 GC terminal and an E301 silicone elastomer (10%)-celite column. Temperatures were increased at 10°C/min up to 250°C. Peaks were detected by flame ionization.

RESULTS

Growth inhibition studies. After the addition of biocide (4 to 20 μg/ml) to actively growing cultures of *E. coli*, growth immediately ceased. Bronopol-induced bacteriostasis persisted for up to 90 min, and when growth was resumed, it was at a lower rate than that of the controls (Fig. 1). The length of the bronopol-induced bacteriostasis was proportional to the applied biocide concentration, as was the degree of growth inhibition measured immediately after recovery from this bacteriostasis. Plots of growth rate inhibition after the induced bacteriostasis against bronopol concentration allowed estimates of the MIC to be made (13 μg/ml; Fig. 1).

The ability of thiols to neutralize and reverse the growth inhibitory action of bronopol was examined by the addition of cysteine to growth-inhibited cultures at various times after the addition of biocide (7 μg/ml). Bronopol concentrations of one-half the MIC were used so that both activation and neutralization of the growth inhibitory effects might be observed. When the molar ratio of cysteine to bronopol was 1:1, cysteine failed to alter the pattern of inhibition. However, at a 10:1 molar ratio the length of the induced bacteriostatic period was substantially reduced provided that the addition of cysteine was made less than 40 min after that of the biocide. In no case was the inhibited growth rate following the shortened bacteriostatic period increased by the presence of a neutralizer (Fig. 2). The addition of the enzyme catalase or superoxide dismutase to the growth-inhibited cultures caused no change in the pattern of inhibition.

Bactericidal activity. Bactericidal activity was approximated to first-order kinetics for concentrations of bronopol greater than 100 μg/ml (Fig. 3a). The compound had a concentration exponent of 0.9 (Fig. 3b) and a temperature coefficient (θ_{10}) of 2.9 (Fig. 3c), and activity increased with increasing pH (Fig. 3d).

Time-survival data were redetermined for bronopol at 500 μg/ml under anoxic conditions and under aerobic conditions in the presence of catalase and superoxide dismutase. All three sets of conditions significantly reduced the degree of bactericidal activity. Such effects were particularly marked under anoxia and in the presence of superoxide dismutase (Fig. 4).

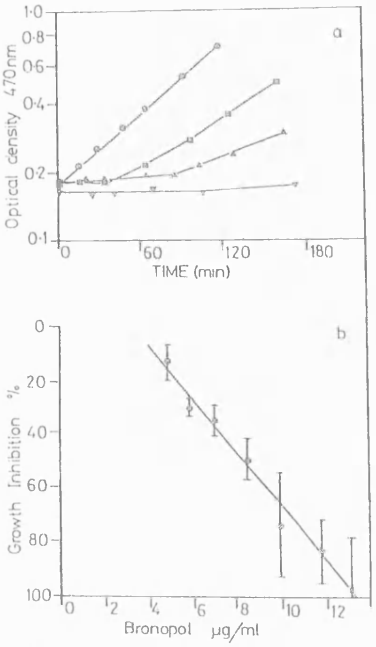

FIG. 1. Growth inhibitory activity of bronopol towards *E. coli* ATCC 8739. (a) Cultures were grown in a simple salt, chemically defined medium until logarithmic phase and $E_{470\ nm}$ of 0.15. Bronopol (●, 0 μg/ml; ■, 5 μg/ml; ▲, 10 μg/ml; ▼, 12.5 μg/ml) was added, and growth was monitored as $E_{470\ nm}$ for up to 2 h. (b) Plot of the inhibition of growth rate relative to untreated controls after the biocide-induced bacteriostatic period versus bronopol concentration for the determination of the minimum growth inhibitory concentration (13 μg/ml).

Reactions of bronopol with thiols. The reactions of bronopol with cysteine, cysteine methyl ester, and glutathione were assessed in D_2O in 5-mm-diameter NMR tubes. The results of these studies were checked by TLC, and when necessary, larger scale reactions were conducted with a view to separate and identify the reaction products.

The results of previous TLC studies suggest that in the presence of air the major reaction product of cysteine and bronopol is cystine (16). This result was readily confirmed with equimolar amounts of the two reactants by using a combination of ^{1}H NMR and TLC, with the additional observation that consumption of cysteine was not accompanied by any measurable loss of bronopol. Even with molar ratios of cysteine to bronopol of 10:1, the NMR signals for bronopol were clearly seen when the cysteine had been completely consumed. In the absence of bronopol, cysteine hydrochloride was essentially unchanged after several hours in solution in an NMR tube. With cysteine methyl ester, essentially similar results were obtained. In this case, the differences in the NMR spectra of the free bases of the thiol and disulfide were more marked, and the conversion was confirmed without the need for TLC. With the methyl ester,

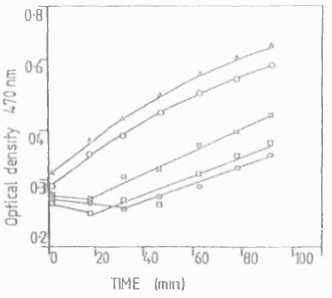

FIG. 2. Effect of cysteine on the growth inhibitory activity of bronopol toward *E. coli* ATCC 8739. Cultures were grown in a simple salt, chemically defined medium until logarithmic phase and $E_{470\ nm}$ of 0.3. Bronopol (7 μg/ml) was added to all cultures except the control (▲), followed by cysteine (62 μg/ml) at various times (○, 0 min; ▨, 20 min; and □, 40 min). ●, No cysteine. Growth was monitored as $E_{470\ nm}$ for up to 2 h.

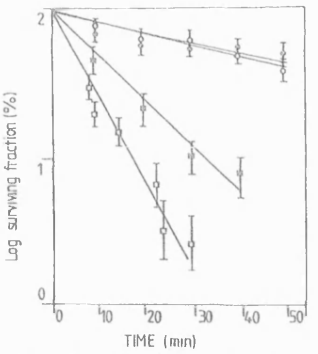

FIG. 4. Bactericidal activity of bronopol (500 μg/ml toward washed suspensions of *E. coli* ATCC 8739 (□) in phosphate-buffered saline (0.1 M, pH 7.0) in the presence of catalase (●, 100 U/ml) or superoxide dismutase (▨, 200 U/ml) and under anoxic conditions (○).

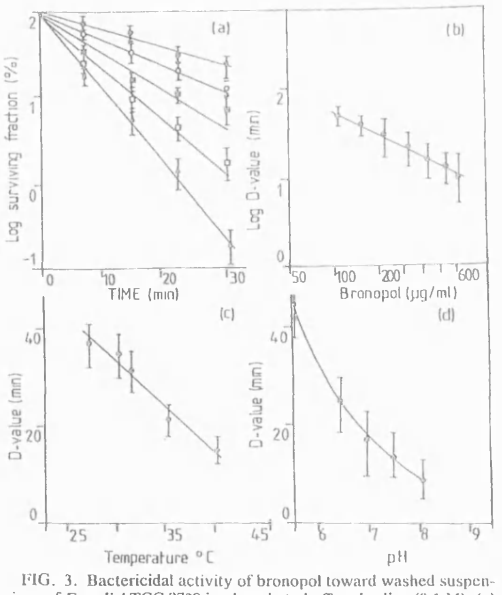

FIG. 3. Bactericidal activity of bronopol toward washed suspensions of *E. coli* ATCC 8739 in phosphate-buffered saline (0.1 M). (a) Time-survival data determined at 35°C and pH 7.0. Bronopol concentrations: ●, 100 μg/ml; ○, 200 μg/ml; ▨, 300 μg/ml; □, 400 μg/ml; and ▲, 500 μg/ml. (b) Plot of decimal reduction times following bronopol treatment (pH 7, 35°C) versus bronopol concentration for the determination of the concentration exponent (0.9). (c) Effect of temperature on the bactericidal activity of bronopol (500 μg/ml) at pH 7.0 (θ_{10} = 2.9). (d) Effect of pH on the bactericidal activity of bronopol (500 μg/ml) at 35°C.

it was possible to make the solution alkaline and to extract the disulfide with chloroform. Comparison of the extract with an authentic sample confirmed that the disulfide was the sole significant reaction product.

At the relatively low field of 80 MHz, the reaction of bronopol with glutathione gave spectra with considerable areas of overlapping peaks that did not permit assignments to be made with confidence. However, comparison with spectra of authentic material suggested that glutathione was oxidized to the disulfide, as were cysteine and cysteine methyl ester. This result was readily confirmed by TLC. Again, the disulfide was the only significant reaction product, with the bronopol remaining unchanged.

In the presence of air, the reactions of bronopol with all the above-mentioned thiols were extremely rapid even at the lower bronopol/thiol ratios and despite the relatively poor aeration that is expected in a 5-mm-diameter NMR tube. The most likely oxidant for consumption in these reactions is oxygen because the bronopol itself clearly plays a catalytic role. Oxygen consumption after mixing bronopol (50 μg/ml in phosphate buffer, 0.1 M, pH 7.0) and cysteine (0.15 mg/ml in phosphate buffer, 0.1 M, pH 7.0) was assessed by conducting the reaction in a metabolic chamber with an oxygen electrode (Rank Bros., Cambridge, United Kingdom) and a strip chart recorder. Immediately after bronopol addition, oxygen was rapidly consumed and a state of anoxia was achieved within 2 min (Fig. 5).

In the absence of air, the reactions took a different course. Two observations were made in the NMR experiments. First, the bronopol was consumed; second, the reactions were considerably more complex than those observed in the presence of air. The reaction was considerably slower than that observed previously, with significant levels of cysteine remaining after 2 h when a bronopol/cysteine ratio of 1:10 was used. In the case of cysteine methyl ester, there was also evidence from both NMR and TLC data for the formation of the disulfide, but this was no longer the exclusive product. In view of the complexity of the reaction, no attempt was made to follow the reaction with glutathione under anoxic conditions. Instead, cysteine methyl ester was used in a relatively large-scale reaction to allow the isolation of at least some of the reaction products.

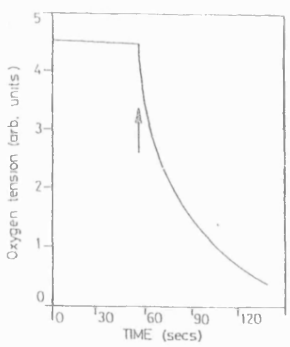

FIG. 5. Oxygen consumption following the interaction of solutions of bronopol (50 μg/ml in phosphate buffer, 0.1 M, pH 7.0) and cysteine (0.15 mg/ml in phosphate buffer, 0.1 M, pH 7.0) in a metabolic chamber with oxygen electrode. Arrow denotes time of bronopol addition. arb., Arbitrary.

When cysteine methyl ester hydrochloride (8.6 g) was treated with bronopol (10 g) in phosphate buffer at pH 7.0 after removal of oxygen by bubbling nitrogen in, the solution became cloudy. After 2 days, a yellow solid and a yellow oil separated. The solid was collected by filtration, and the oil was removed by solvent extraction. The solid was insoluble in all common solvents. Mass spectrometry showed the sequential loss of 32 mass units, and simple chemical tests confirmed the solid to be sulfur.

The oil was a complex mixture, but flash chromatography followed by GC-mass spectometry and two-dimensional NMR allowed the identification of the most volatile components. These components were two closely related isomers, which at first were difficult to separate on GC, and were not physically separable for NMR analysis. They were therefore examined as a mixture, and the NMR spectrum in $CDCl_3$ at 300 MHz showed resonances that were assigned as follows: δ 1.64, CH_3; δ 1.81, CH_3; δ 2.02, impurity; δ 2.82 to 3.31, a complex region of multiplets due to nonequivalent protons of CH_2 coupled to CH; δ 3.67, 3.73, 3.74, and 3.75, four OCH_3 groups; δ 4.0, CH; and δ 4.32, CH. The ^{13}C NMR spectrum was easier to interpret and showed very clearly that the sample was a mixture of two very similar compounds. The spectrum showed the following resonances in $CDCl_3$: δ 24.96 (CH_3), 27.78 (CH_3), 38.53 (CH_2), 39.62 (CH_2), 51.99 (OCH_3), 52.64 (OCH_3), 65.12 (CH), and 65.31 (CH). The multiplicities of hydrogen resonances (CH, CH_2 and CH_3) were determined by the DEPT pulse sequence.

A two-dimensional (HETCOR) NMR experiment confirmed the assignments and correlated the proton multiplets at δ 4.0 and 4.32 with the carbon resonances at δ 65.12 and 65.31, respectively. The proton and carbon resonances for the methoxy groups correlated as expected. The carbon with signal at δ 39.62 correlated with multiplets and δ 3.3 and 2.8 in the proton spectrum, and the carbon with signal at δ 38.53

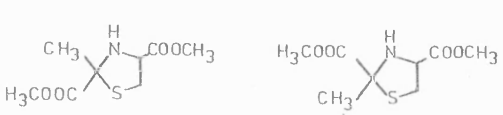

FIG. 6. Proposed structures for the anoxic reaction products of cysteine and bronopol.

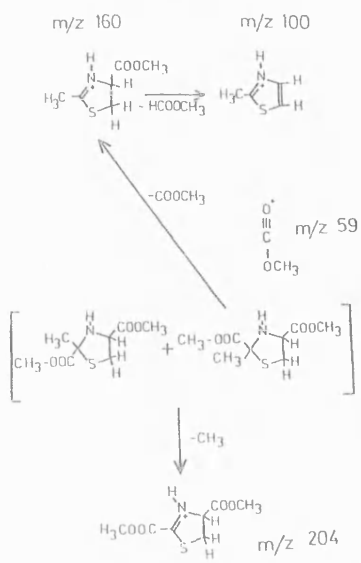

FIG. 7. Proposed fragmentation pattern for the anoxic reaction products of cysteine and bronopol subjected to GC-mass spectrometry.

correlated with multiplets at δ 3.3 and 3.0 in the proton spectrum. The carbon resonances at δ 27.78 and 24.96 of the remaining methyl groups correlated with proton resonances at δ 1.64 and 1.81, respectively.

These observations are entirely consistent with the *cis* and *trans* structures given in Fig. 6. The fragmentation pattern after GC-mass spectrometry was also consistent with the proposed structures. Although there was no molecular ion, there was a small peak at *m/z* 204, representing the loss of a CH_3 group, and the remainder of the anticipated fragmentation pattern was easily reconciled (Fig. 7). Structures for smaller peaks at *m/z* 119 and 87 have not yet been assigned.

The formation of thiazolidines has been observed when aqueous solutions of cystine are exposed to air for prolonged periods (5). Presumably, the reaction proceeds by the oxidation of cystine to pyruvic acid and the condensation of pyruvic acid with a molecule of cysteine (Fig. 8), which is also formed by the breakdown of cystine.

In the present study, a reaction involving the methyl ester gave reaction products analogous to those depicted in Fig. 6. Presumably, bronopol acts as an oxidizing agent in the absence of oxygen, although no breakdown products of bronopol were isolated from the very complex reaction mixture.

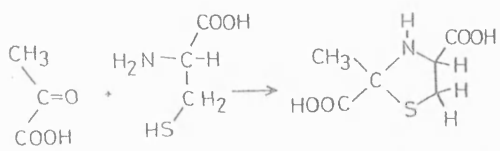

FIG. 8. Thiazolidine formation by condensation of pyruvic acid and cysteine.

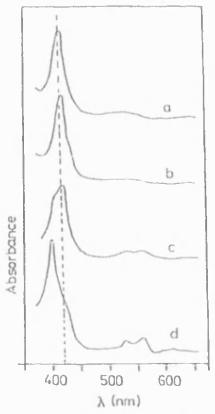

FIG. 9. Absorption spectra of cytochrome *c* (2 μg/ml in phosphate buffer, 0.1 M, pH 7.0) alone (a) and following addition of bronopol (200 μg/ml) (b) or cysteine (100 μg/ml) (c) or the simultaneous addition of bronopol and cysteine (d).

Cytochrome *c*. The absorption spectra of cytochrome *c* (2 μg/ml in phosphate buffer, 0.1 M, pH 7.0 [Fig. 9a]) were determined with a Pye Unicam SP8-500 spectrophotometer following addition of bronopol (200 μg/ml [Fig. 9b]) or cysteine (100 μg/ml [Fig. 9c]) or both (Fig. 9d). The addition of bronopol alone caused no change in the cytochrome *c* spectrum, but the addition of cysteine caused a gradual shift in the spectrum, indicating the formation of the reduced form of cytochrome *c* (Fig. 9c). Simultaneous addition of bronopol and cysteine caused an immediate change in the cytochrome *c* spectrum to that of the reduced form (Fig. 9d).

DISCUSSION

Chemical studies of the interactions of bronopol with cysteine, cysteine methyl ester, and glutathione demonstrated that in the presence of air bronopol acts as a catalyst for the oxidation of thiol groups to disulfides, with the rapid consumption of oxygen. The observation that bronopol was not consumed in this reaction allows an extension of the reaction mechanism proposed by Stretton and Manson (16), according to which a radical anion intermediate is formed from bronopol during the conversion of cysteine to cystine and of glutathione to its disulfide (Fig. 10). NMR spectroscopy is a more convenient and more informative technique for observing such reactions than TLC because it unambiguously shows that bronopol is not destroyed while thiol is consumed. In cell suspensions, such catalysis leads to an alteration in the redox state, oxidation of glutathione to its disulfide, and inhibition of enzyme function and growth. These consequences of catalysis are evidenced by the immediate cessation of growth on bronopol addition to actively growing cultures and the generation of anoxic conditions. The length of the induced bacteriostatic period depended on bronopol concentration and was shortened by the addition of exogenous thiols such as cysteine. This observation suggested that there is a second slower reaction that did not require oxygen and that consumed or neutralized the bronopol within the cell. This neutralization occurred at a rate that depended on the concentration of accessible thiols. Presumably, restoration of cellular function follows bronopol consumption and occurs through the resumption of respiration and the reduction of glutathione disulfide to glutathione via glutathione reductase. Reductions in the rate of growth, relative to those of control cultures, following the induced bacteriostasis probably reflect irreversible damage to the cell caused by bronopol treatment, possibly through the generation of oxygen radicals (6) during the catalytic period (Fig. 10) and from the continued diffusion of oxygen into the suspensions during bacteriostasis. When the slower reactions of bronopol with thiols were examined by maintaining anoxia, a number of reaction products other than the disulfide were, indeed, produced and bronopol was consumed. The production of elemental sulfur in the reaction of bronopol with cysteine methyl ester indicates an oxidative degradation of the cysteine derivative that is comparable with the slow autoxidation of cysteine observed in air (5). In this study, the oxidation of the thiol occurred in the absence of oxygen and presumably resulted in the chemical reduction of bronopol. The reaction was complex, and the observed products in this work should not be taken as reflective of the products formed in vivo under anoxic conditions.

The bactericidal activity of bronopol was expressed at concentrations greatly in excess of the MIC and was suppressed by the absence of oxygen or the presence of catalase or superoxide dismutase. The estimated concentration exponent (0.92) agreed with that reported by Hurwitz and McCarthy (9). The activity of bronopol increased with increasing pH from 5.5 to 8.0. This result is consistent with the published data (17) and suggests that the presence of the thiolate anion contributes to the reactivity of the thiol (10). The enzymes superoxide dismutase and catalase scavenge

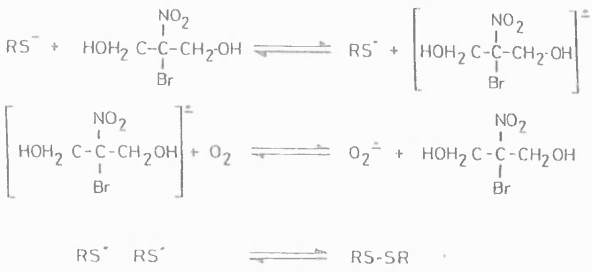

FIG. 10. Formation of radical anion intermediate from bronopol.

peroxide and superoxide from suspension supernatants (8). The protection against the bactericidal activity of bronopol afforded by these enzymes suggests that the activity stems from the aerobic interaction and the generation of active oxygen species from oxygen diffusing into the suspensions during bronopol treatment. Such reactions would act as a continuous source of superoxide for as long as bronopol remained. The generation of superoxide by the aerobic reaction of bronopol with thiols was demonstrated by the reduction of cytochrome *c* by superoxide (Fig. 9) (2, 7).

The results therefore suggest a dual action of bronopol, with catalytic oxidation of accessible thiols being responsible for the growth inhibition and generation of free radicals causing cell death. The extent of killing reflects the number of free radicals generated from oxygen diffusing into the suspension during the biocide-induced bacteriostasis and therefore relates to bronopol concentration and the length of the bacteriostatic period.

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LITERATURE CITED

1. Al-Hiti, M. M. A., and P. Gilbert. 1980. Changes in preservative sensitivity for the USP antimicrobial agents effectiveness test microorganisms. J. Appl. Bacteriol. 49:119–126.
2. Bernofsky, C., and S. C. Wanda. 1983. Reduction of cytochrome c by hydrogen peroxide and its inhibition by superoxide dismutases. Biochem. Biophys. Res. Commun. 111:231–238.
3. Bryce, D. M., B. Croshaw, J. E. Hall, V. R. Holland, and B. Lessel. 1978. The activity and safety of the antimicrobial agent bronopol (2-bromo-2-nitropropan-1,3-diol). J. Soc. Cosmet. Chem. 29:3–24.
4. Croshaw, B., M. J. Groves, and B. Lessel. 1964. Some properties of bronopol, a new antimicrobial agent against *Pseudomonas aeruginosa*. J. Pharm. Pharmacol. 16:127T.
5. Dann, J. R., G. L. Oliver, and J. W. Gates. 1957. The reaction of cystine and lanthionine with aqueous calcium hydroxide. The identification of 2-methylthiazolidine-2,4-dicarboxylic acid. J. Am. Chem. Soc. 79:1644–1649.
6. Fridovich, I. 1978. The biology of oxygen radicals. Science 201: 875–880.
7. Ginsberg, C. L., and J. Everse. 1978. Studies on the reduction of cytochrome c by thiols. Bioorganic Chem. 7:481–492.
8. Gregory, E. M., and I. Fridovich. 1973. Oxygen toxicity and the superoxide dismutases. J. Bacteriol. 114:1193–1197.
9. Hurwitz, S. J., and T. J. McCarthy. 1985. Dynamics of disinfection of selected preservatives against *Escherichia coli*. J. Pharm. Sci. 74:892–894.
10. Jocelyn, P. 1972. Biochemistry of the SH group. Academic Press, Inc. (London), Ltd., London.
11. Russell, G. A., and W. C. Danen. 1967. Electron transfer processes. VIII. Coupling of radicals with carbanions. J. Am. Chem. Soc. 90:347–353.
12. Saito, H., and T. Onoda. 1974. Antibacterial action of bronopol on various bacteria, especially on *Pseudomonas aeruginosa*. Chemotherapy (Tokyo) 22:1466–1473.
13. Smith, I., and J. G. Feinberg. 1972. Paper and thin layer chromatography and electrophoresis, 2nd ed. Longman, London.
14. Stahl, E. 1969. Thin layer chromatography, 2nd ed. Springer-Verlag KG, Berlin.
15. Storrs, F. J., and D. E. Bell. 1983. Allergic contact dermatitis to 2-bromo-2-nitropropan-1,3-diol. J. Am. Acad. Dermatol. 8:157–170.
16. Stretton, R. J., and T. W. Manson. 1973. Some aspects of the mode of action of the antibacterial compound bronopol (2-bromo-2-nitropropan-1,3-diol). J. Appl. Bacteriol. 36:61–76.
17. Wong, W. C., and T. F. Preece. 1985. *Pseudomonas tolasi* in mushroom (*Agaricus bisporus*) crops: activity of formulations of 2-bromo-2-nitropropan-1,3-diol (bronopol) against the bacterium and the use of the compound to control blotch disease. J. Appl. Bacteriol. 58:275–281.
18. Zolsnai, T. 1961. Versuche zur Entdeckung neuer Fungistatika. II. Nitro-verbindungen. Biochem. Pharmacol. 5:287–293.

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NOTES

Peak Selection through Orthogonal Focusing

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The majority of NMR spectroscopists have abandoned the off-resonance decoupling technique for determining the number of protons attached to each carbon in a ^{13}C spectrum, in favor of techniques such as APT and DEPT which provide better signal-to-noise and greater clarity of presentation (*1*). A feature of both these techniques is that CH_2 signals can be obtained with opposite phase from CH and CH_3 peaks, thereby increasing the information content of the spectrum. At relatively low field, however, there is a reasonable probability of peak overlap in a crowded spectrum which may cause confusion. At 20.1 MHz, for example, the ^{13}C spectrum of cholesteryl acetate contains two examples of complete and one partial overlap. If the overlapping peaks happen to have opposite phases, the nulling which results is very misleading.

It is possible, using DEPT, to achieve subspectra of individual CH, CH_2, and CH_3 carbons by manipulation of data in memory. In theory this should overcome the overlap problem, but in practice the data manipulation is only convenient where the machine is supplied with suitable software, and the manipulated subspectra are sometimes ambiguous, depending on the quality of the original data: there are drawbacks inherent in only presenting manipulated data. Eschewing manipulation, quaternary carbons may be selected (*2*) or CH carbons alone may be obtained with DEPT. The technique described here gives "even" and "odd" subspectra as required but, unlike earlier ways of achieving this, results in good nulling of unwanted signals, uses only one memory block, and does not require data manipulation. By taking advantage of nuclear Overhauser enhancements and slow relaxation rates for quaternary carbons the "even" spectrum can usually be reduced to CH_2 only. The "odd" spectrum provides useful support for DEPT, in cases where there is doubt.

The basis of the technique is the production of orthogonally focused signals for carbons with even and odd numbers of attached protons. With reference to the rotating frame (Fig. 1) following a 90° carbon pulse and comparing only CH and CH_2 for simplicity, it is apparent that after a delay of $1/4J$ the lines will have progressed as in (A). At this point 180° pulses for both ^{13}C and ^{1}H will result in double interchange of "fast" and "slow" components, so that after a further delay of $1/4J$ the lines will have reached the position in (B) and will be focused. This point has been recognized as a means of producing a subspectrum of quaternary carbons only (*3*). It is possible to impose a further 90° carbon pulse (with either x or y phasing) to rotate either the even or odd signals into the z direction, giving (C). A delay of $1/2J$ reunites the

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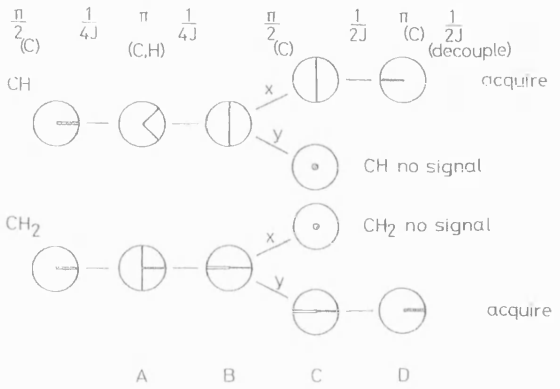
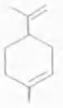


FIG. 1. Diagrammatic representation of orthogonal focusing followed by selection of "odd" or "even" multiplicities.

signal components as in (D). A 180° ^{13}C pulse followed by a further delay of $1/2J$ with broadband ^{1}H decoupling focuses the signals for recording, giving either a C + CH_2 or CH + CH_3 subspectrum depending on the phase of the second 90° carbon pulse.

Spectra obtained using both *x* and *y* pulses at stage B in Fig. 1 are shown in Fig. 2 for limonene,



along with the normal broadband-decoupled spectrum (a). Limonene is a good test of this kind of technique, since it contains sp^3 CH, CH_2, and CH_3, sp^2 CH and CH_2, and quaternary carbons. The compound is also cheap, is readily available, and can be used almost neat. The quaternary carbon peaks in Fig. 2c can be virtually eliminated by reducing the relaxation delay, or enhanced by increasing it.

In the rare instances of compounds where quaternary carbons undergo rapid longitudinal relaxation, this observation does not apply and quaternaries may be observed as relatively intense signals owing to their minimal proton coupling and insensitivity to the *J* values assumed in the sequence: such carbons are readily identified using DEPT.

Coupling constants which are different from the assumed value result in incomplete nulling owing to imperfect alignment of the unwanted signals in the *z* direction: the result is an out-of-phase signal of reduced intensity which is readily distinguished, and in practice the method is not unduly sensitive to differences between coupling constants. An analysis of unwanted residuals from misset delays shows that the intensity of the unwanted lines has essentially a $\sin^2$ relationship to the difference in angle

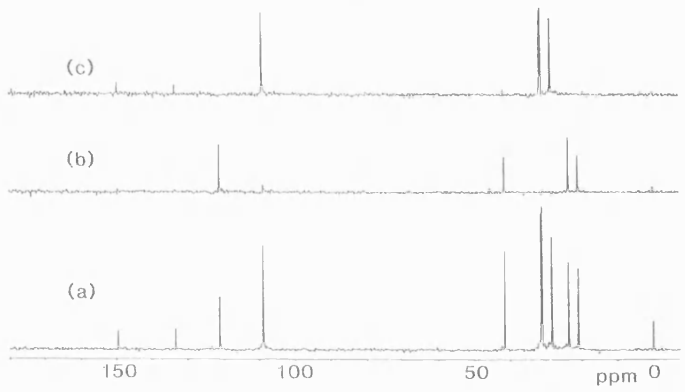


FIG. 2. Broadband-decoupled ^{13}C spectrum of limonene using 90° pulses; (b) CH + CH_3 subspectrum obtained using an x pulse at stage B in Fig. 1; (c) C + CH_2 subspectrum obtained using y pulse at stage B in Fig. 1. All three spectra were obtained at 20.1 MHz in absolute intensity mode with 24 scans, 2 s relaxation delay. In (b) and (c) $1/4J$ was set to 0.0017 s. The sample contained sufficient deuterochloroform for a lock signal and about 5% tetramethylsilane; quadrature phase cycling was used throughout.

at the orthogonal focusing point from the desired 90°. As an example, for a carbon atom attached to one proton a ±10% difference from the assumed 90° is calculated to result in a residual line intensity of only about 2%. The residual intensities are calculated to be greatest for methyl carbons, owing to the greater angular error on the outer lines when the coupling constant is poorly matched. It may therefore be best to set the delays to suit the coupling constants for sp^3 carbons, although in practice it does not matter very much. The efficient nulling for methine carbons contrasts with the situation in the normal J-modulated spin-echo experiment (*4*).

For routine application this technique has provided reliable support for DEPT, particularly for the occasional spectrum where the nulling produced by DEPT is worse than usual or where CH_2 and CH_3 chemical shifts are very close. The spectra retain nuclear Overhauser enhancements if the decoupler is kept on during the relaxation delay. As a general observation, for spectra obtained with the same pulse widths and relaxation delays, signal-to-noise is usually about 60% of that obtained in a broadband-decoupled spectrum.

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REFERENCES

1. J. K. M. SANDERS AND B. K. HUNTER, "Modern NMR Spectroscopy," Oxford Univ. Press, Oxford, 252, 1987.
2. M. R. BENDALL, D. T. PEGG, D. M. DODDRELL, S. R. JOHNS, AND R. I. WILLING, *J. Chem. Soc., Chem. Commun.*, 1138 (1982).
3. M. R. BENDALL, D. M. DODDRELL, AND D. T. PEGG, *J. Am. Chem. Soc.* **103**, 4603 (1981).
4. F.-K. PEI AND R. FREEMAN, *J. Magn. Reson.* **48**, 318 (1982).

Mechanisms of Chemical Reactions with Biomolecules

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Chemical antimicrobial agents exert their effect upon bacterial cells either through irreversible covalent associations with biomolecular targets resulting in adjunct formation or through reversible interactions of a physico-chemical nature which alter the physical chemistry of the target molecule. In the former instance, an understanding of the mechanisms and range of chemical interactions between biocide and potential targets is crucial to the elucidation of the mechanism of action in the intact cell (Gilbert 1985). Traditional methods for the evaluation of the chemical mechanism of action of reactive antimicrobial compounds have tended to involve two separate stages following exposure of cells to the agent. The first stage is one of separation and purification of the reaction products, often involving chromatography, and the second is the application of spectroscopy of various kinds to determine the molecular structure of the adjuncts. While these methods are still useful, it is becoming possible to carry out direct spectroscopic studies on systems *in vivo* and *in vitro* which are quicker, easier and more elegant and informative. The principal technique for such studies is nuclear magnetic resonance (NMR) spectroscopy, which in recent years has made substantial advances in sensitivity, with accompanying detailed improvements in experimental technique to enhance the information content of the spectra. Potential users of the technique, who have a basic grounding in NMR spectroscopy, will find the recent books by Sanders & Hunter (1987) and Derome (1987) particularly informative with respect to the latest developments. We propose to illustrate the usefulness of NMR spectroscopy as a tool for understanding the mode of action of biocides by reference to *in vitro* studies backed up by more traditional approaches (in the case of bronopol and *iso*thiazolones) and also by reference to direct *in vivo* methodologies (for noxythiolin and taurolin).

Mechanisms of Action of Chemical Biocides

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Bronopol (2-bromo-2-nitro-propan-1,3-diol)

Bronopol has a broad spectrum of antibacterial activity (Saito & Onoda 1974) and is widely used, at concentrations up to 0.1%, as a preservative of pharmaceutical and cosmetic products (Croshaw *et al* 1964; Storrs & Bell 1983). There have been a number of studies of the mechanism of action of bronopol, all of which conclude that activity relates to interaction with essential thiols within the cell (Stretton & Manson 1973; Bryce *et al*. 1978; Wong & Preece 1985). Such interaction is thought to lead to oxidation of thiols through a radical anion intermediate (Russell & Danen 1967). Unlike other thiol-interactive antimicrobial agents (e.g. thiomersal), bronopol possesses significant bactericidal activity which cannot be explained solely in terms of thiol oxidation.

Earlier studies on the mode of action have shown that in aqueous solution cysteine is rapidly oxidized to cystine in the presence of bronopol (Stretton & Manson 1973). This experiment is easy to repeat, by using thin-layer chromatography as the analytical method. In such systems, however, bronopol is not easy to detect and is even harder to quantify by this means. When the bronopol–cysteine reaction is carried out inside the spectrometer (Fig. 1) it is possible, not only to follow the disappearance of the cysteine and the formation of cystine but also to observe the bronopol quantitatively (Shepherd *et al*. 1988). The spectra in Fig. 1 show very clearly that the bronopol is scarcely consumed in this reaction, while the cysteine is rapidly oxidized. When the reactions were monitored by oxygen electrode it was apparent that the reaction mixture, and indeed bacterial suspensions, were rapidly depleted of oxygen upon bronopol addition, irrespective of the bronopol: thiol ratio. The bronopol is therefore acting as an oxidative catalyst in the presence of oxygen.

In the absence of air (Fig. 2) the reaction is much slower and the bronopol disappears at a comparable rate to the cysteine (Shepherd *et al*. 1988). While the reaction is far too complex to allow the direct determination of the structures of the reaction products, the experiment does indicate that different modes of action must apply under oxic and anoxic conditions. Subsequent work with cysteine methyl ester, involving solvent extraction and chromatographic separation of the reaction products, showed that the thiol had been extensively degraded, under anaerobic conditions, by an oxidative mechanism to elemental sulphur. In this case the bronopol itself must be chemically reduced since there is no oxygen available.

Such chemical information is compatible with the observed biological effects of bronopol. Following the addition of bronopol to actively growing cultures of bacteria, growth ceases immediately for a period dependent upon the concentration applied. After this induced bacteriostasis, growth proceeds at an inhibited rate. Bactericidal concentrations (100–500 μg/ml) are considerably in excess of the minimum growth inhibitory concentration (MIC; 10–15 μg/ml). Bactericidal activity, but not growth inhibitory actions, may be

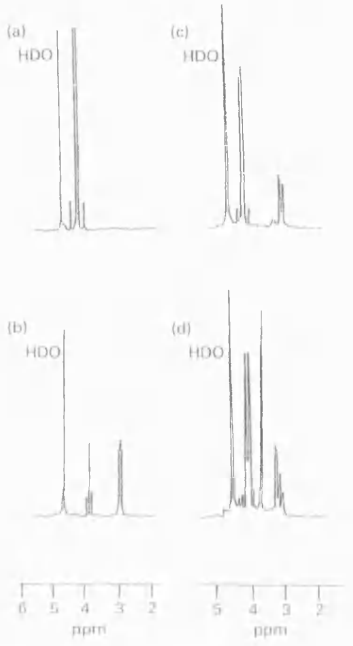


FIG. 1. 80-MHz ^{1}H NMR spectra in deuterium oxide of; (a) bronopol, (b) cysteine, (c) equimolar mixtures of bronopol (20 mg) and cysteine (17 mg) reacted, in air at room temperature, within the NMR tube, and (d) cystine. Note that (c) shows the continued presence of the bronopol doublet whilst cystine is a major product. (For a detailed assignment of the peaks readers are directed to Shepherd *et al.* 1988.)

significantly reduced by the exclusion of air or inclusion of the enzymes catalase or superoxide dismutase in cell suspensions. It is apparent that bronopol undergoes two distinct types of reaction with accessible thiols within cells. Under aerobic conditions, bronopol catalyses the oxidation of thiols such as glutathione and cysteine to their disulphides, utilizing atmospheric oxygen as the oxidant and generating an anoxic state. Further oxygen diffusing into the system will be used catalytically in this manner so long as bronopol remains. Such reactions will bring about the immediate cessation of bacterial growth. By-products or intermediates in this aerobic reaction are active oxygen species such as superoxide and peroxide. These are directly responsible for

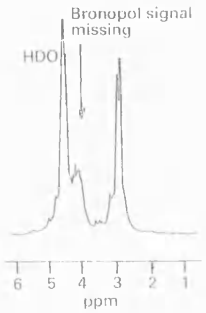


FIG. 2. 80-MHz ^{1}H NMR spectrum of equimolar mixtures of bronopol (20 mg) and cysteine (17 mg) in deuterium oxide reacted in the absence of air at room temperature in the NMR tube. Note the loss of the bronopol doublet and the formation of a new product which does not resemble cysteine or cystine (see Fig. 1).

the bactericidal activity of the compound and for the reduced rate of growth following the bacteriostatic period. Once an anoxic state has been created in the reaction mixture it will be maintained by the presence of unreacted bronopol and reduced thiols but it will allow the slower, consumptive reactions of bronopol to predominate until all the bronopol is consumed. Only then may enzymes such as glutathione reductase restore the redox state of the cell and allow growth to resume. Whether this occurs depends upon the extent of free radical damage incurred during the interim period.

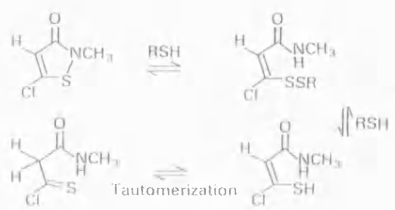
*Iso*thiazolone Biocides

*Iso*thiazolone biocides such as ben*zisoth*iazolone (BIT), N-methyl *iso*thiazolone (MIT) and 5-chloro-N-methyl*iso*thiazolone (Cl–MIT) are widely used as environmental biocides. BIT has been shown to interact oxidatively with accessible thiols and specifically to inhibit glucose transport *in vivo* (Fuller *et al.* 1985). Addition of thioglycollate, mercaptoethanol, glutathione or cysteine at equimolar concentrations to all three biocides totally neutralizes their inhibitory effects. For Cl–MIT activity is also reversed *in vivo* by the addition of valine or histidine.

Conventional spectral and analytical studies have suggested that the reactions of BIT with thiols are as outlined below. Initial reaction leads to the formation of a disulphide conjugate. Reaction of the conjugate with further thiols leads to the formation of a thiol dimer and a ring-opened form of the biocide which itself could serve as a further source of interactive thiol to

give a dimerized biocide as an additional product (Fuller *et al.* 1985). Cl–MIT and MIT are thought to act similarly to BIT but whilst BIT is a hypersensitizing agent, Cl–MIT is both a primary skin irritant (>25 μg/ml; Weaver *et al.* 1985) and Ames positive (Monte *et al.* 1983). Cl–MIT has reported bactericidal activity, unlike BIT which is primarily bacteriostatic in action. Cl–MIT is antibacterial at considerably lower concentrations than BIT. The reasons behind these fundamental differences in activity were unclear until NMR spectroscopy studies were undertaken.

Reactions of BIT with cysteine were examined by ^1H NMR spectroscopy with a Bruker WP80 spectrometer and the reaction pathway suggested by Fuller *et al.* (1985) confirmed. When similar studies were undertaken with Cl–MIT, however, (Fig. 3) loss of the downfield proton peak indicated exchange with D_2O and suggested that the ring-opened form of the biocide could tautomerize, to give a highly reactive thio-acid chloride.



Formation of the thio-acid chloride *in vivo* would explain the apparently anomalous toxicological and antimicrobial activity of the chlorinated *iso*thiazolone in that it would possess a far higher reactivity than the parent molecule.

Noxythiolin and Taurolin

Investigations into these and other 'masked formaldehyde' antibacterial agents can be conceptually separated into those studies which deal with the nature of the active antibacterial form and those dealing with the detoxification processes possessed by the organism. In both cases the critical experiments have been conducted using NMR spectroscopy, either for direct observation of chemical equilibria, for which it was superior to alternative methods, or for *in vivo* observation of metabolites, a task for which NMR is uniquely suited.

The toxic intermediates

On a simplistic basis both noxythiolin and taurolin can be considered as

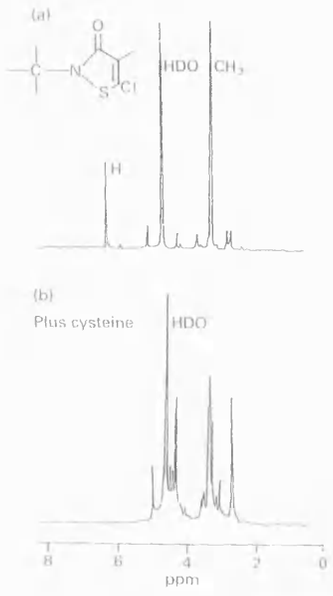
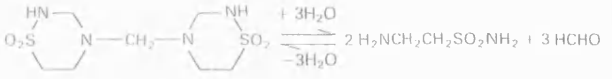


FIG. 3. 80-MHz ^1H NMR spectra of, (a) 5-chloro-N-methyl *iso*thiazolone, and (b) equimolar mixtures of 5-choloro-N-methyl *iso*thiazolone (5 mg) and cysteine (10 mg) in deuterium oxide reacted at room temperature in the NMR tube. Note the loss of the downfield proton of 5-chloro-N-methyl *iso*thiazolone indicating exchange with the D_2O solvent and therefore isomerization.

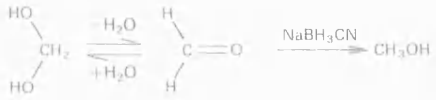
formaldehyde-producing antibacterial agents, the overall simplified equations for their aqueous decomposition being as described in the following schemes.

Noxythiolin

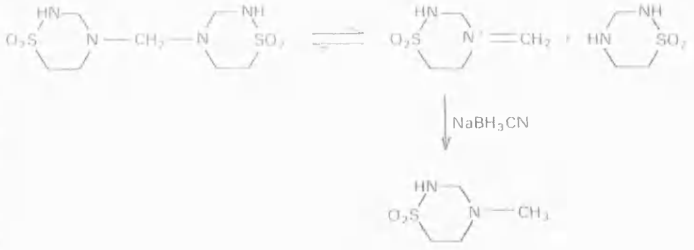


Taurolin

A simple chemical experiment (Gidley *et al.* 1981; Gidley & Sanders 1983) demonstrates an important difference in the chemical behaviour of the two compounds. Sodium cyanoborohydride is a reducing agent which is reasonably stable in aqueous solution, and which reacts slowly with formaldehyde to give methanol. (It should be noted that formaldehyde exists largely as the hydrated form in aqueous solution.)



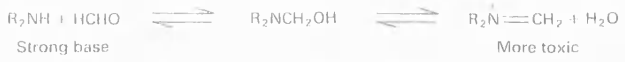
When a solution of noxythiolin is treated with reducing agent the only reaction is the slow reduction of formaldehyde to methanol. The reaction tends to show that noxythiolin is truly a source of formaldehyde as the toxic species and it is no surprise that *Escherichia coli* survival curves, after exposure to noxythiolin solutions, are very close to those obtained with formaldehyde itself (Gidley & Sanders 1983). In contrast, treatment of taurolin solutions with the reducing agent gives almost quantitative conversion to an N-methyl intermediate.



The methylene iminium ion $R_2N^+{=}CH_2$ is a more reactive species than formaldehyde towards hydride ions provided by the reducing agent, so that the reaction is chemically quite orthodox, proving the presence of the iminium ion. NMR spectroscopy provides a convenient means of following the reaction

as it proceeds without the necessity for repetitious chemical analyses (Gidley *et al.* 1981).

We now have an explanation for the enhanced antibacterial activity of taurolin and for the increased antibacterial activity of formaldehyde in the presence of certain amines. The iminium ion is likely to be more readily attacked than formaldehyde by the biological nucleophile, in an analogous manner to the attack by hydride ions, resulting in more rapid inactivation of the susceptible bacterial system. For systems in which the amine is added separately, the distinction can be made between weak and strong bases, in an exactly comparable way.



In these studies the major role of NMR spectroscopy is as an elegant, non-disruptive, simultaneous assay for formaldehyde and other reaction products or intermediates. An illustration of the extraordinary power of the method, however, is provided in the case of imidazole–formaldehyde mixtures (Gidley & Sanders 1983).

This system does not produce iminium ions in great quantity but was none the less more effective than formaldehyde alone. Another NMR spectroscopy experiment showed that the above equilibrium was capable of producing naked (non-hydrated) formaldehyde about 40 times more frequently than a simple aqueous solution of formaldehyde alone, thus providing a feasible explanation for the enhanced antibacterial activity.

Metabolic studies

In order to study metabolic reactions involving potentially toxic species it is necessary to use concentrations below those which kill the cell. The pathways which are revealed are thus essentially detoxification processes and probably compete with those through which the antibacterial agent exerts its desired effects. Whether comparable studies of the toxic reactions will ever be feasible remains to be seen.

In vivo ^{13}C NMR spectroscopy

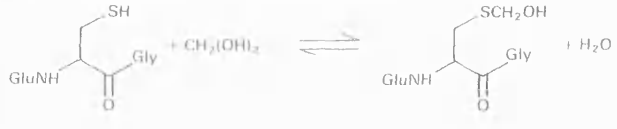
While there is no room here for a general discourse on NMR spectroscopy, some explanation of the special attributes of ^{13}C NMR spectroscopy will help to explain how the experiments with formaldehyde were conceived.

One of the most significant features of ^{13}C NMR spectroscopy, compared to ^{1}H NMR as used in the work described previously, is that ^{13}C is a minor isotope, constituting only 1.1% of natural carbon. If the material whose metabolism is to be studied is labelled with a high concentration of ^{13}C, the peaks for the added material are of greater intensity and can be studied with minimal interference from background natural abundance carbon. In addition, the range of resonance frequencies for ^{13}C is very wide, so that interference between peaks is less likely, even for chemically similar molecules, and the exact resonance frequency (chemical shift) is highly characteristic for any given position in a molecule, provided that pH is held constant. Given that carbon–carbon coupling is not a problem (owing to the low natural abundance), that each carbon resonance can be reduced to a single line, and that efficient techniques are available for the determination of the number of protons attached to the labelled carbon, it can thus be seen that ^{13}C NMR spectroscopy is tailor-made for the problem in hand.

Formaldehyde metabolism by Escherichia coli

The work described by Sanders and co-workers (Hunter *et al.* 1984, 1985; Mason *et al.* 1986) examines this subject in great depth, and includes a consideration of deuterium incorporation which can also be measured and provides further insight. The major findings, however, can be simply stated as follows.

A ^{13}C spectrum of an *E. coli* suspension, treated with ^{13}C-labelled formaldehyde at a sublethal concentration, initially shows only one major peak, corresponding to $^{13}CH_2(OH)_2$, the hydrated form of formaldehyde, at δ83.2 (Fig. 4a). Three hours later there are five other peaks corresponding to methanol, formate and three rather similar products, namely propane-1,2 diol, propane-1,3-diol and glycerol (Fig. 4b), the latter identified with the aid of bacteria grown on ^{13}C-labelled glucose. Subsequent experiments showed that the first-formed adduct is 5-(hydroxymethyl)-glutathione, formed non-enzymically within a very short time after formaldehyde addition.



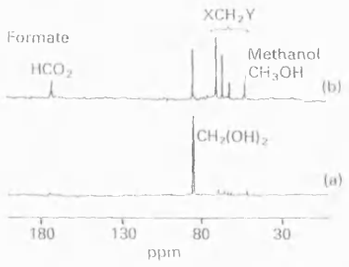


FIG. 4. 100.6-MHz ^{13}C NMR spectrum in deuterium oxide of a non-aerated *E. coli* suspension (10^{12} cells/ml) initially containing 10 mmol/l ^{13}C-labelled formaldehyde. (a) Spectrum obtained during the first 20 min after formaldehyde addition, and (b) 3 h later. A total of 500 transients was collected, with a 2-s relaxation delay and 45° pulse width. (Reproduced with permission from Hunter *et al.* 1984.)

This glutathione adduct, with a labelled ^{13}C resonance at δ66.6, remains until the one-carbon residue is disposed of by metabolism. There is no evidence for incorporation of the label into serine, methionine or other methyl groups, which effectively rules out the incorporation of this exogenous formaldehyde into the tetrahydrofolate pool. Deoxygenation virtually abolishes metabolism, while aeration results in loss of label, presumably through oxidation to bicarbonate and loss of carbon dioxide.

While the identification of metabolites such as methanol and formate follows very simply from measurement of the chemical shift and determination of the number of protons attached to the labelled carbon, the identity of the similar three-carbon products is less easy to establish. The chemical shifts of the labelled carbons are similar at δ68.0, 63.9 and 59.8; all three of these have two attached protons with CH-coupling constants of about 140 Hz. All three are therefore consistent with the unit $R^{13}CH_2OH$. This problem was solved quite elegantly by feeding the bacteria with glucose, uniformly labelled with ^{13}C at a level of 14.3%, compared to the 90% labelling of the formaldehyde. In the spectra obtained it is therefore possible to distinguish carbons arising from glucose (14.3% label) from those arising from formaldehyde (90% label), and to infer the diverse origins of carbons with intermediate levels of labelling. The more immediate intention, however, was to allow the unidentified carbons, labelled from ^{13}C formaldehyde, to interact with their neighbours, which will now be seen in the spectrum. This reveals their chemical shift and gives a clue as to their chemical environment. This concept may be expressed as follows for propane-1,2-diol, one of the previously identified metabolites

(*C=90% label, °C=14% label). Using ^{12}C glucose the ^{13}C NMR spectrum shows

$$HO{*}CH_2 - CHOH - CH_3.$$

(CHOH-CH_3 is of low relative intensity)

Using partially ^{13}C glucose shows

$$HO{*}CH_2 - {^\circ}CHOH - {^\circ}CH_3.$$

*C and °C are coupled, i.e. they each cause splitting of the others' signal. We therefore know the chemical shift of the carbon adjacent to *C and the number of protons attached to it; a great aid to identification. Similar arguments apply to glycerol and propane-1,3 diol, with some subtleties which cannot be expounded here.

While ^{13}C NMR is almost ideal for studies of formaldehyde metabolism, there are limitations to the technique imposed mainly by the inherent lack of sensitivity associated with this nucleus. Even at the relatively high field strength used by Sanders and co-workers, spectrum accumulation normally took 20 min to give acceptable signal-to-noise. In a rapidly changing system this would be too slow. Nevertheless, it is likely that the use of NMR spectroscopy to investigate the mode of action of antibacterial agents will become more common and even more effective.

References

Bryce, D.M., Croshaw, B., Hall, J.E., Holland, V.R. & Lessel, B. 1978. The activity and safety of the antimicrobial agent bronopol (2-bromo-2-nitropropan-1,3-diol). *Journal of Cosmetic Chemistry* **29**, 3–24.

Croshaw, B., Groves, M.J. & Lessel, B. 1964. Some properties of bronopol, a new antimicrobial agent against *Pseudomonas aeruginosa*. *Journal of Pharmacy and Pharmacology* **16**, 127T.

Derome, A.E. 1987. *Modern NMR Techniques for Chemistry Research*. Oxford: Pergamon Press.

Fuller, S.J., Denyer, S.P., Hugo, W.B., Pemberton, D., Woodcock, P.M. & Buckley, A.J. 1985. The mode of action of 1,2 benzisothiazolin-3-one on *Staphylococcus aureus*. *Letters in Applied Microbiology* **1**, 13–15.

Gidley, M.J. & Sanders, J.K.M. 1983. Mechanisms of antibacterial formaldehyde delivery from noxythiolin and other 'masked-formaldehyde' compounds. *Journal of Pharmacy and Pharmacology* **35**, 712–717.

Gidley, M.J., Sanders, J.K.M., Myers, E.R. & Allwood, M.C. 1981. The mode of antibacterial action of some 'masked' formaldehyde compounds. *FEBS Letters* **127**, 225–227.

Gilbert, P. 1985. The revival of microorganisms sub-lethally injured by chemical inhibitors. In *The Revival of Injured Microbes*, pp. 175–197, eds Andrew, M.H.E. & Russell, A.D., London: Academic Press.

Hunter, B.K., Nicholls, K.M. & Sanders, J.K.M. 1984. Formaldehyde metabolism by *Escherichia coli*. *In vivo* carbon, deuterium and two dimensional NMR observations of multiple detoxifying reactions. *Biochemistry* **23**, 508–514.

Hunter, B.K., Nicholls, K.M. & Sanders, J.K.M. 1985. Formaldehyde metabolism by

Escherichia coli. Carbon and solvent deuterium incorporation into glycerol, 1,2-propanediol and 1,3-propanediol. *Biochemistry* **24**, 4148–4155.

MASON, P.R., SANDERS, J.K.M., CRAWFORD, A. & HUNTER, B.K. 1986. Formaldehyde metabolism by *Escherichia coli*. Detection by *in vivo* ^{13}C NMR spectroscopy of *s*-(hydroxymethyl) glutathione as a transient cellular intermediate. *Biochemistry* **25**, 4504–4507.

MONTE, W.C., ASHOOR, S.H. & LEWIS, B.J. 1983. Mutagenicity of two non-formaldehyde-forming antimicrobial agents. *Food Chemistry and Toxicology*, **21** 695–697.

RUSSELL, G.A. & DANEN, W.C. 1967. Electron transfer processes VIII. Coupling of radicals with carbanions. *Journal of the American Chemical Society* **90**, 347–353.

SAITO, H. & ONODA, T. 1974. Antibacterial action of bronopol on various bacteria, especially on *Pseudomonas aeruginosa*. *Chemotheraphy, Tokyo* **22**, 1466–1473.

SANDERS, J.K.H. & HUNTER, B.K. 1987. *Modern NMR Spectroscopy*. Oxford: Oxford University Press.

SHEPHERD, J.A., WAIGH, R.D. & GILBERT, P. 1988. Antibacterial action of 2-bromo-2-nitropropan-1,3-diol (bronopol). *Journal of Bacteriology* **32**, 1693–1698.

STORRS, F.J. & BELL, D.E. 1983. Allergic contact dermatitis to 2-bromo-2-nitropropan-1,3 diol. *Journal of the American Academy of Dermatologists* **8**, 157–170.

STRETTON, R.J. & MANSON, T.W. 1983. Some aspects of the mode of action of the anti-bacterial compound bronopol (2-bromo-2-nitro propan-1,3-diol). *Journal of Applied Bacteriology* **36**, 61–76.

WEAVER, J.E., CARDIN, C.W. & MAIBACH, H.I. 1985. Dose-response assessments of kathon biocide (1) Diagnostic use and diagnostic threshold patch testing with sensitised humans. *Contact Dermatitis* **12**, 141–145.

WONG, W.C. & PREECE, T.F. 1985. *Pseudomonas tolasii* in mushroom (*Agaricus bisporus*) crops: Activity of formulations of 2-bromo-2-nitropropane-1,3-diol (bronopol) against the bacterium and the use of this compound to control blotch disease. *Journal of Applied Bacteriology* **58**, 275–281.

Journal of Applied Bacteriology 1990, **69**, 578–584 3728/01/90

Chemical reactivity of some isothiazolone biocides

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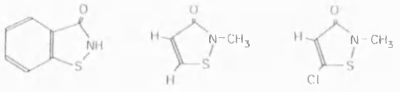
Chemical reactions between the isothiazolone biocides, N-methylisothiazol-3-one (MIT), benzisothiazol-3-one (BIT) and 5-chloro-N-methylisothiazol-3-one (CMIT) with cysteine have been investigated by u.v. and NMR spectroscopy. At physiological pH all three agents interacted oxidatively with thiols to form disulphides. Further interaction with thiols caused the release of cystine and formation of a reduced, ring-opened form of the biocide (mercaptoacrylamide). In an analogous fashion to the initial reaction the mercaptoacrylamide reacted with another molecule of biocide to give biocide dimers. NMR spectral studies indicated that for CMIT the mercaptoacrylamide form is capable of tautomerization to a highly reactive thio-acyl chloride. Formation of mercaptoacrylamide was in all cases highly pH-dependent. Alcohol dehydrogenase was insensitive to all three agents but was highly sensitive to CMIT when co-administered with dithiothreitol. Capacity to form a thioacyl chloride from the mercaptoacrylamide is suggested to account for much of this enhanced activity. Stopped-flow spectroscopic studies showed rates of reaction with glutathione (GSH) to directly parallel antimicrobial activity. Additionally, CMIT was able to react directly with both ionization states of GSH (pH 7–10) whilst BIT and MIT appeared only to interact when the glutamyl-nitrogen of GSH was charged (pH 8·5).

Isothiazolone biocides such as benzisothiazolone (BIT, I), N-methylisothiazolone (MIT, II) and 5-chloro-N-methylisothiazolone (CMIT, III) are widely used as industrial biocides (Singer 1976; Andrykovitch & Neihof 1987). Mixtures of CMIT and MIT (Kathon, Rohm & Haas Inc.) are also used in the preservation of cosmetic products (Zeelie & McCarthy 1983; Law *et al.* 1984, 1987).



I II III

All three agents are strongly antagonized by exogenous thiol-containing agents and are thought to interact oxidatively with accessible thiols, such as glutathione, within the cell (Fuller *et al.* 1985; Collier *et al.* 1990). Whilst BIT is a skin sensitizing agent, CMIT is both a primary skin irritant (> 25 μg/ml; Weaver *et al.* 1985) and Ames positive (Monte *et al.* 1983). Earlier studies (Collier *et al.* 1990) also show that CMIT, but not BIT and MIT, is neutralized by the addition of histidine and valine and produces patterns of growth inhibition and associated morphological changes which are reminiscent of inhibition of initiation of DNA replication.

The chemical reactions of MIT and CMIT with thiols have not been previously reported. Fuller *et al.* (1985), however, identified S-(2-carbamoylphenylthio)-γ-L-glutamyl-L-cysteinylglycine (VI, Fig. 1) as an initial by-product of the interaction between glutathione (GSH) and

† Corresponding author.

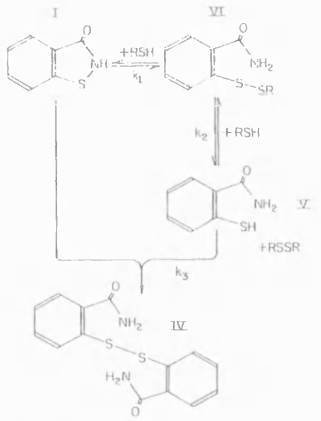


Fig. 1. Reaction pathway proposed by Fuller (1986) for the interaction of glutathione (RSH) with benziso-thiazolone (I).

BIT and suggested that BIT reacted with most thiol-containing compounds to form disulphide adjuncts. Further interaction with thiols caused the release of oxidized thiol dimers and a reduced, ring-opened form of BIT (mercaptobenzamide, V, Fig. 1). In an analogous fashion to the initial reaction with thiol the mercaptobenzamide was able to provide further sources of thiol for the unreacted biocide to give BIT dimers (IV, Fig. 1). Results described in the previous paper (Collier *et al.* 1990) suggest CMIT reacted differently from non-chlorinated isothiazolones such as BIT and that such differences might be associated with the special toxicological problems associated with this compound. The present paper reports on such chemistry.

Materials and Methods

CHEMICALS

Benzisothiazolone (Proxel, ICI plc Organics Division) was kindly donated by ICI plc Organics Division (Manchester, UK). 5-chloro-N-methylisothiazolone and N-methylisothiazolone were prepared according to the methods described by Lewis *et al.* (1971). Glutathione, NAD and cysteine were obtained from Sigma and purified yeast alcohol dehydrogenase (ADH) from Boehringer Mannheim (FDR). All other reagents were of the purest available grade and obtained from BDH (UK).

NMR SPECTROSCOPIC STUDIES OF ISOTHIAZOLONE/THIOL INTERACTIONS

N-Methylisothiazolone (5 mg) and CMIT (5 mg) were allowed to react at room temperature with cysteine (10 mg) in deuterated water (D_2O). Reactions were carried out in 5 mm NMR tubes. Cysteine was used instead of GSH as a source of thiol, as the interpretation of its NMR spectrum is less complicated. After 5 min, proton-NMR (^{1}H-NMR) spectra were determined using a Bruker 80 MHz spectrometer.

ASSAY FOR YEAST ALCOHOL DEHYDROGENASE ACTIVITY

Phosphate buffer (25°C, 50 mmol/l, pH 7·0), ethylenediaminetetracetic acid (EDTA, 30 mmol/l), NAD (0·4 mmol/l) and purified yeast alcohol dehydrogenase (0·01 units) were mixed in a quartz cuvette. Reactions were started by the addition of ethanol (0·2 ml, 96%), after pre-incubation for 1 min with biocide (0–20 μmol/l) and dithiothreitol (0–35 μmol/l). Preliminary experiments had determined that in the absence of biocide these conditions gave zero-order kinetics over an incubation period of > 5 min. Reactions were monitored by the increase in optical density ($A_{340\ nm}$).

REACTIONS OF GLUTATHIONE WITH ISOTHIAZOLONE BIOCIDES

u.v. absorption spectra (250–400 nm) were determined for solutions of BIT (175 μmol/l), MIT (110 μmol/l) and CMIT (90 μmol/l) and also for mixtures (3 : 1 molar ratio, GSH : isothiazolone) of these solutions with GSH (phosphate buffer, 50 mmol/l, pH 6·0–8·0). Absorption maxima were observed at 300, 275 and 272 nm for BIT, MIT and CMIT, respectively. These did not alter with pH. On reaction with GSH, solutions of BIT decreased in their absorption at 300 nm and gave new absorption maxima at 270 and 340 nm. Both of the new peaks became more marked as pH increased to pH 8·0. Fuller (1986) used reverse phase HPLC

and appropriate standards and showed that these spectral peaks arose from the formation of a mercaptobenzamide (V, Fig. 1). In the present study and that of Mattisek & Lehnguth (1987), MIT was seen to behave similarly with a new absorption maximum appearing at 300 nm, also corresponding to a mercaptoacrylamide. This pattern was not repeated by CMIT which gave distinctive peaks at pH 6·0 (245 and 300 nm) and pH 8·0 (265 and 305 nm). At physiological pH the peak produced at 305 nm was most prominent. Whilst it was unlikely that any observable product of the CMIT reaction corresponded to a mercaptoacrylamide the product formed at 305 nm was selected as a suitable indicator of reaction rate for stopped-flow spectrometry.

Rates of reaction between GSH and the isothiazolone biocides in phosphate buffer (50 mmol/l; pH 7–10) were determined by stopped-flow spectroscopy (Model SF-3A, Nortech Laboratories, Canterbury, UK) and the techniques described by Dawber & Moore (1980). The ionic strength of the reaction mixtures was kept constant by the addition of potassium chloride. Reactions were studied at 25°C under pseudo first-order conditions with isothiazolone concentrations kept constant (5×10^{-4} mol/l) and very low in relation to those of GSH ($2{\cdot}0 \times 10^{-2}$–$1{\cdot}6 \times 10^{-1}$ mol/l) which were varied. Preliminary experiments indicated that at these values, isothiazolone concentrations did not influence reaction rates determined at 275 nm (BIT), 300 nm (MIT) and 305 nm (CMIT). Buffers, containing EDTA (10^{-4} mol/l), used for stopped-flow experiments were bubbled with N_2 for 5 min before use to minimize oxidative destruction of GSH during the studies. For stopped-flow studies, rate constants were average values taken from a minimum set of four individual runs.

Results and Discussion

CHEMISTRY OF ISOTHIAZOLONE BIOCIDES WITH CYSTEINE

Proton NMR spectra indicate that MIT (Fig. 2a) reacts with cysteine to give a single reaction product together with some residual unreacted cysteine. Chemical shift of the CH_2 peak in the product (δ 3·2–3·3) from that of cysteine (δ 2·9–3·0) suggests that the products are simple adjuncts with MIT (Fig. 2b) and analogous to structure VI (Fig. 1). This suggestion is supported by AM1 (Dewar *et al.* 1985) and CNDO/2 (Pople & Beveridge 1970) calculations of charge distribution within MIT which show the sulphur as the most likely site for nucleophilic attack (S, +0·0209; C_5, −0·0004; N, −0·1708). NMR spectra determined under similar conditions for CMIT (Fig. 4) were complex and indicated a number of different reaction products which were not characterized further. Significantly, the resonance corresponding to the 4′ hydrogen of CMIT (δ 6·24) was no longer apparent in the spectrum after reaction with cysteine. The results of AM1 (Dewar *et al.* 1985) and CNDO/2 (Pople & Beveridge 1970) calculations of charge distribution for CMIT suggest that both C_5 (+0·0754) and the S—N (S, +0·0453; N, −0·175) bond are likely to be susceptible to nucleophilic attack. Attack at C_5 would lead to loss of chlorine and formation of

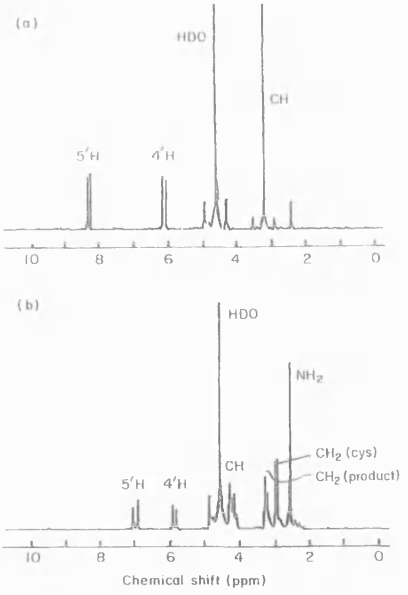


Fig. 2. ^{1}H-NMR spectra for (a) N-methylisothiazolone (II) and (b) mixtures of N-methylisothiazolone (5 mg) with cysteine (10 mg).

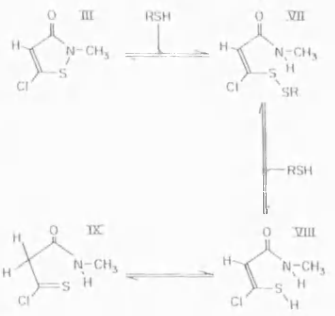


Fig. 3. Proposed pathway for the interaction of 5-chloro-N-methylisothiazolone (III) with cysteine (RSH).

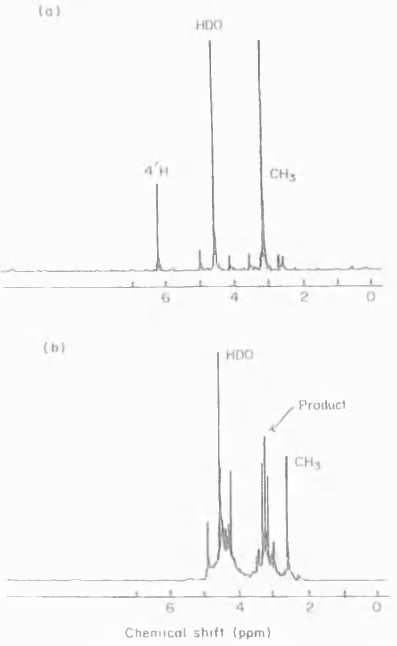


Fig. 4. ^{1}H-NMR spectra for (a) 5-chloro-N-methylisothiazolone (III) and (b) mixtures of 5-chloro-N-methylisothiazolone (5 mg) with cysteine (10 mg).

a stable C-S product, without the possibility of exchange of the 4'H with solvent (Fig. 4b).

The alternative reaction route is similar to that for MIT and BIT and involves nucleophilic attack at the S N bond to form a mixed disulphide (VII, Fig. 3). Further reaction with cysteine would then, as before, lead to the release of a mercaptoacrylamide (VIII, Fig. 3). In the case of CMIT, the mercaptoacrylamide would be unstable and tautomerize to give a thioacyl chloride (IX, Fig. 3) and loss of the alkene proton (4'H) in NMR spectra. Indeed, *ab initio* calculations, using the methods of Dewar *et al.* (1985) for the thioacyl chloride, show it to be the preferred tautomer with an energy state 2·42–6·69 Kcal/mol less than that of the mercaptoacrylamide (VIII). Thioacyl chlorides are highly reactive chemicals and will provide strong electrophilic centres which would interact not only with thiols but also with amines, water, etc. (Fig. 5). The existence of thioacyl chlorides *in vivo* would be short-lived so their effects upon cellular proteins, nucleic acids, etc., whilst marked, would be severe only in the immediate vicinity of their bioformation. Indeed formation of such acylating agents is likely to account for the increased antimicrobial activity and unexpected toxicity associated with Kathon and CMIT.

Absence of a readily accessible thiol in the enzyme yeast alcohol dehydrogenase (ADH) renders it relatively insensitive to classical thiol-interactive agents. If the especial activity of CMIT is caused by activation to mercaptoacrylamide and subsequently to a thioacyl chloride, then activity of CMIT towards ADH ought to be significantly increased by simultaneous incubation with a source of thiol.

Dithiothreitol provides a source of thiol, as does cysteine, but without an associated amine which might otherwise quench the reactivity of thioacyl chloride. The effects of various concentrations of CMIT (0–20 μmol/l), MIT (0–2 mmol/l) and BIT (0–0·5 mmol/l) on ADH activity were assessed in the presence of various concentrations of dithiothreitol (0–35 μmol/l). In the absence of dithiothreitol all three compounds were relatively inactive towards this enzyme ($ID_{50\%}$ 0·5–2 mmol/l). After pre-incubation of enzyme with isothiazolone and dithiothreitol, activity was enhanced significantly (Fig. 6) for CMIT but not for MIT or BIT. The degree of activation of CMIT increased with increasing concentration of dithiothreitol.

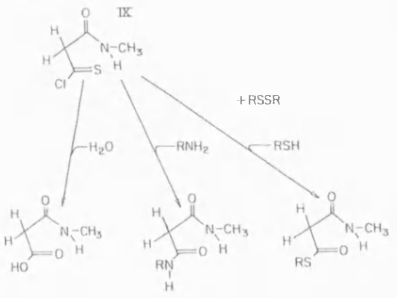


Fig. 5. Proposed pathway for the interactions of thioacyl chloride (IX) with water, amines and thiols.

REACTION OF GLUTATHIONE WITH ISOTHIAZOLONE BIOCIDES

Rates of reaction of the isothiazolone biocides with glutathione were monitored by stopped-flow spectroscopy at 305 nm. Numerical analysis of the data collected by stopped-flow spectroscopy enabled determination of first-order reaction rate constants (k_1), where:

$$k_{obs} = k_1[GSH][drug] \quad (1)$$

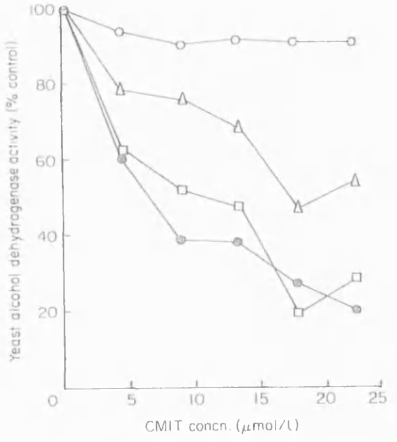


Fig. 6. Inhibition of yeast alcohol dehydrogenase by 5-chloro-N-methylisothiazolone when pre-incubated with various concentrations of dithiothreitol. ○, zero; △, 8·5 μmol/l; □, 17 μmol/l and ●, 35 μmol/l.

and since drug concentration was kept so low as to be negligible

$$k_{obs} = k_1[GSH] \quad (2)$$

Observed reaction rates (k_{obs}) were derived from simple first-order reaction kinetics for BIT and MIT and, after correction for a steady upward drift in the absorbance at 305 nm with time for CMIT, over the time-course of the stopped-flow experiment.

Pseudo-first-order rate constants for these reactions closely paralleled antimicrobial activity, being 20 times greater for CMIT ($1{\cdot}053 \times 10^3$/mol/s) than for BIT ($4{\cdot}883 \times 10^1$/mol/s) and 100 times greater than for MIT ($1{\cdot}077 \times 10^1$/mol/s). This relates to estimates of minimum growth inhibitory concentration of 14 and 18 μg/ml for BIT, 41 and 240 μg/ml for MIT and 0·5 and 0·1 μg/ml for CMIT towards *Escherichia coli* and *Schizosaccharomyces pombe* respectively (Collier *et al.* 1990).

Dependence of reaction rate upon pH in the range pH 7–10 was determined at a single glutathione : isothiazolone molar ratio (200 : 1) as k_{obs}. Glutathione possesses seven different ionization states. Only two of these involve thiol group ionization (Douglas 1988). These ions have pKa values of 8·93 (GSH[A]) and 9·08 and 9·28 (GSH[B]). Each of these species is nucleophilic and this will lead to a composite sigmoidal pH reaction rate profile (Douglas 1988) in the range pH 7–10. Reaction rate pH profiles for the isothiazolones (Fig. 7) clearly show bell-shaped curves for MIT and BIT and a composite sigmoidal curve for CMIT.

The sigmoidal dependance of k_{obs} for CMIT (Fig. 7c) gives a value of $pK_{app} = 8{\cdot}4$. This is close to the value of the pKa for the –SH group of GSH (Douglas 1988) and in line with attack by the GS^- form of glutathione on CMIT. Attack by GS^-, as opposed to GSH, is common in reactions between GSH and other thiols (Douglas 1988). The bell-shaped curve for BIT (Fig. 7a) indicates that at least two ionizations are affecting the observed kinetics. By analogy with the other systems, the GS^- form is likely to be the kinetically reactive form of glutathione. The rate decreases at higher pH indicates that an additional feature is decreasing the rate at higher pH. The simplest explanation of this is that BIT ionizes, according to equation (3), and that the attack by GS^- upon the conju-

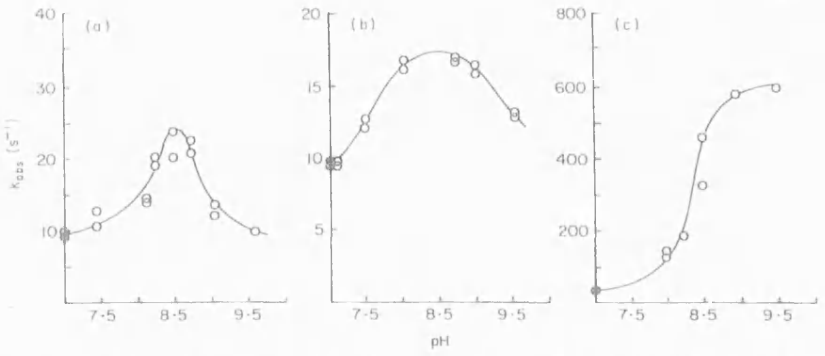


Fig. 7. Interdependence of pH and observed reaction rate of glutathione ($2{\cdot}5 \times 10^{-3}$ mol/l) with (a) benzisothiazolone (5×10^{-4} mol/l, I), (b) N-methylisothiazolone (5×10^{-4} mol/l, II) and (c) 5-chloro-N-methylisothiazolone (5×10^{-4} mol/l, III) in phosphate buffer (50 mmol/l, 25°C).

gate base (below) is less than that upon BIT (*I*).

(3)

The rate of reaction would then decrease at higher pH. However, MIT (II) with an N-CH_3 site blocking such an ionization still has a bell-shaped pH profile. We have also found that N-methylated BIT reacts similarly to BIT with a bell-shaped pH profile.

If the full rate equation were derived for Fig. 1 it would contain k_2 and k_3. The amount of RSH reacting (i.e. GSH) would depend on its pKa (i.e. GSH $-$ GS^-) but the reaction rate would also depend on the mercaptoacrylamide (V) and reflect its fate also. This species is the tautomer of an unstable thioaldehyde where the tautomerism is highly likely to be acid : base catalysed. This was considered as a possible explanation of the rate decreases at higher pH. NMR spectra (Fig. 2), however, show that the product of the reaction, unlike that for CMIT, is not deuterium-exchanged at position 4 (α to the sulphur) indicating that keto : thioenol equilibration had not occurred for MIT nor BIT reactions. The decrease in rate at higher pH presumably reflects partitioning of the mercaptoacrylamide (k_2 and k_3 processes) or the decrease in concentration of V by disulphide formation which is expected to become rapid at higher pH values as commonly found for thiol oxidations. The disulphide does not absorb light at 305 nm and would not therefore directly contribute to k_{obs}.

5-chloro-N-methylisothiazolone does not give the rate decrease at higher pH values. This is probably because the mercaptoacrylamide in this instance (VIII) is rapidly destroyed by thiolysis and hydrolysis after rapid tautomerization to the thioacyl chloride (Figs 3 and 5).

This and the previous paper (Collier *et al.* 1990) demonstrate the especial reactivity of the chlorinated isothiazolones (CMIT) and suggest possible interactions of these at the level of DNA synthesis. At physiological pH, interaction of thiols with the —S—N— bond of the isothiazolone ring is likely to predominate in all cases. In the case of CMIT this interaction leads to increases in biological activity, through the formation first of a mercaptoacrylamide and subsequently a highly reactive thio-acyl chloride tautomer. Such a reaction mechanism might explain the reported Ames positivity and potent skin-sensitizing properties of CMIT-containing compounds.

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References

Andrykovitch, G. & Neihof, R.A. 1987 Fuel soluble biocides for control of *Cladosporium resinae* in hydrocarbon fuels. *Journal of Industrial Microbiology* **2**, 35–40.

Collier, P.J., Ramsey, A.J., Austin, P. & Gilbert, P. 1990 Growth inhibitory and biocidal activity of some isothiazolone biocides. *Journal of Applied Bacteriology* **69**, 569–577.

Dawber, J.G. & Moore, A.T. 1980 In *Chemistry for the Life Sciences*, 2nd edn. pp. 89–92. London: Macmillan Press.

Dewar, M.J.S., Zoebisch, E.G., Healy, E.F. & Stewart, J.J.P. 1985 AM1: A new general purpose quantum mechanical molecular model. *The Journal of the American Chemical Society* **107**, 3902–3909.

Douglas, K.T. 1988 Reactivity of glutathione in model systems. In *Glutathione Conjugation: Mechanisms and Biological Significance* eds. Sies, H. & Ketterer, B. pp. 1–41. London: Academic Press.

Fuller, S.J. 1986 The mode of antibacterial action of 2-thiocyanato-benzamide and some of its derivatives. PhD Thesis, University of Nottingham.

Fuller, S.J., Denyer, S.P., Hugo, W.B., Pemberton, C., Woodcock, P.M. & Buckley, A.J. 1985 The mode of action of 1,2-benzisothiazolin-3-one on *Staphylococcus aureus*. *Letters in Applied Microbiology* **1**, 13–15.

Law, A.B., Moss, J.N., & Lashen, E.S. 1984 Kathon CG: A new single component broad spectrum preservative system for cosmetics and toiletries. *Cosmetics Science Technology Series* **1**, 129–141.

Law, A.B., Donnelly, T.W. & Lashen, E.S. 1987 Evaluation of methylchloroisothiazolone/methylisothiazolone and other antimicrobial agents as preservatives for home and industrial products. *Prodotto Chimica Aerosol Selezione* **28**, 27–31.

Lewis, S.N., Miller, A., Hausman, M. & Szamborshi, E.C. 1971 Isothiazoles 1: 4-isothiazolin-3-ones. A general synthesis from 3,3'-dithiodipropionamides *Journal of Heterocyclic Chemistry* **8**, 571–580.

Mattisek, R. & Lehnguth, R. 1987 Zür analytik mikrobicider isothiazolone V. *Fresenius Zeitung Analytiker Chemisch* **328**, 108–111.

Monte, W.C., Ashoor, S.H. & Lewis, B.J. 1983 Mutagenicity of two non-formaldehyde forming antimicrobial agents. *Food Chemistry and Toxicology* **21**, 695–697.

Pople, J.A & Beveridge, D.L. 1970 *Approximate Molecular Orbital Theory*. New York: McGraw Hill.

Singer, M. 1976 Laboratory procedures for assessing the potential of antimicrobial agents as industrial biocides. *Process Biochemistry* July/August, 30–35.

Weaver, J.E., Cardin, C.W. & Maibach, H.I. 1985 Dose response assessments of kathon biocide. *Contact Dermatitis* **12**, 141–145.

Zeelie, J.J. & McCarthy, T.J. 1983 Antioxidants—multifunctional preservatives for cosmetic and toiletry formulations. *Cosmetics and Toiletries* **98**, 51–55.

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COMMUNICATIONS

^{1}H NMR as an analytical tool for the investigation of hydrolysis rates: a method for the rapid evaluation of the shelf-life of aqueous solutions of drugs with ester groups

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Abstract—The rate of hydrolysis of esters of primary and secondary alcohols can be determined quickly and easily by ^{1}H NMR in aqueous solution, provided that the water signal is suppressed by the WATR (Water Attenuation by T_2 Relaxation) method. To evaluate this approach, Arrhenius plots have been constructed for hydrolysis of acetylcholine, carbachol and atropine, and the effect of pH on the hydrolysis of procaine has been determined over a limited range. The results agree well with literature values for rate constants.

The advantages and disadvantages of NMR as an analytical tool are well known. Among the main advantages are the high information content of NMR spectra and the simplicity of method development compared with chromatographic procedures. A major consideration in the routine use of ^{1}H NMR, however, is the necessity for the solvent to have a low ^{1}H concentration. For example, if the sample has a high water content, the ^{1}H signal from the solvent will swamp all the solute resonances. Many methods are available to suppress the water signal, but almost without exception part of the spectrum around the water frequency is lost.

A recent method (known as WATR, water attenuation by T_2 relaxation) for water suppression involves the use of a chemical agent, usually an ammonium or guanidinium salt, to accelerate transverse (spin-spin) relaxation of exchangeable protons (Rabenstein et al 1985; Rabenstein & Fan 1986; Dickinson et al 1987). With a spin-echo pulse sequence the water signal can then be removed from the spectrum while solute signals are in most cases relatively little affected. This is of particular value where the solute signals of interest lie under or close to the water peak. Such is the case with ester groups, where the hydrogen attached to carbon adjacent to oxygen resonates in the δ 4–5·5 region. For studies of ester hydrolysis this resonance is most important, as it is closest to the reactive centre and shows the greatest chemical shift difference when bond cleavage occurs. It is thus the ideal reporter group for hydrolysis studies, provided that the overlying water signal can be selectively suppressed.

We chose two types of experiment to evaluate the method, both having literature results for comparison. We examined first the effect of pH on the hydrolysis of procaine and then we obtained Arrhenius plots for the hydrolysis of acetylcholine, carbachol and atropine at single pH values.

Materials and methods

A solution of the ester (0·5% w/v for acetylcholine chloride, 1% w/v for carbachol chloride, atropine sulphate and procaine hydrochloride) was placed in a jacketed vessel through which hot water was circulated to maintain the desired temperature (see Tables 1–5). Distilled water was used as the solvent except for procaine where phosphate buffer (0·25 M) was employed. pH was adjusted and maintained as required using a pH stat, pumping NaOH (0·5 or 1·0 M). Samples (3·5 mL) were withdrawn at the beginning and at measured intervals, each sample being immediately frozen in liquid nitrogen and kept frozen until required for measurement.

Immediately before measurement the sample was thawed at room temperature. To this solution was added a solution (0·5 mL) containing the internal standard, phosphate buffer (0·1 M) where this was not already present, deuterium oxide (5% v/v) for an NMR lock signal and guanidine hydrochloride (1·0 M). The solution was adjusted to pH 7·3 using 0·5 or 1·0 M NaOH. For procaine and carbachol the internal standard was acetamide (0·05% w/v), but for acetylcholine and atropine the standard used was sodium 3-trimethylsilylpropanesulphonate (0·025 and 0·05% w/v, respectively) to avoid overlapping peaks in the NMR spectrum. The spectra were obtained on a Bruker WP80 spectrometer operating at 80 MHz using a standard Carr-Purcell-Meiboom-Gill (CPMG) spin-echo sequence with a cumulative delay sufficient to cause adequate water suppression, in the range 0·5–1·5 s. For each spectrum 32 scans were accumulated for acetylcholine, carbachol and atropine, 64 scans for procaine.

The integral for the appropriate hydrogen(s) was obtained electronically, normalized with reference to the internal standard, and the logarithm of this value plotted against time to obtain the apparent first order rate constant, the slope being obtained by computerized curve-fitting. For carbachol, atropine and procaine the proton adjacent to the ester oxygen was used to monitor degradation as described above. This procedure could also have been used for acetylcholine, but in this case there was sufficient separation between the acetyl group chemical shifts before and after hydrolysis for these to be integrated separately, giving a slightly better signal to noise ratio.

Results

The first-order rate constants for ester hydrolysis are shown in Tables 1–4. Activation energies and frequency factors are given in Table 5.

A typical plot of ln proton integral (for the proton adjacent to the ester oxygen) against time is shown for atropine in Fig. 1, with examples of typical spectra from which the data were obtained in Fig. 2 and the resulting Arrhenius plot in Fig. 3.

Discussion

Ester groups are common and predictable sites of drug degradation in aqueous solution. The present method offers a means of

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Table 1. First order rate constants for hydrolysis of procaine at 80°C and varying pH.

pH	k(obs) (min^{-1})	log k	s.d.	log k*
8·0	$1·893 \times 10^{-2}$	−1·723	0·080	−1·785
7·8	$1·643 \times 10^{-2}$	−1·784	0·064	
7·7	$1·672 \times 10^{-2}$	−1·777	0·076	
7·5	$1·148 \times 10^{-2}$	−1·940	0·077	−2·047
7·0	$4·638 \times 10^{-3}$	−2·334	0·034	−2·433
6·75	$3·934 \times 10^{-3}$	−2·405	0·062	−2·656
6·5	$1·568 \times 10^{-3}$	−2·805	0·039	−2·890
6·0	$5·589 \times 10^{-4}$	−3·253	0·048	−3·376
5·5	$3·167 \times 10^{-4}$	−3·499	0·067	−3·871
5·0	$4·514 \times 10^{-5}$	−4·345	0·044	−4·369

* Calculated from the data of Higuchi et al (1950).

Table 2. First order rate constants for hydrolysis of acetylcholine at pH 7·0.

Temperature (°C)	k(obs) (min^{-1})	log k	s.d.
45	$3·766 \times 10^{-3}$	−2·424	0·064
50	$6·803 \times 10^{-3}$	−2·167	0·047
60	$2·298 \times 10^{-2}$	−1·639	0·068
65	$3·760 \times 10^{-2}$	−1·425	0·099

Table 3. First order rate constants for hydrolysis of carbachol at pH 8·5.

Temperature (°C)	k(obs) (min^{-1})	log k	s.d.
70	$4·219 \times 10^{-3}$	−2·375	0·046
80	$1·538 \times 10^{-2}$	−1·813	0·048
90	$6·865 \times 10^{-2}$	−1·163	0·071

Table 4. First order rate constants for hydrolysis of atropine at pH 8·0.

Temperature (°C)	k(obs) (min^{-1})	log k	s.d.
60	$2·952 \times 10^{-3}$	−2·530	0·038
70	$9·655 \times 10^{-3}$	−2·015	0·050
80	$2·019 \times 10^{-2}$	−1·695	0·051
88	$4·945 \times 10^{-2}$	−1·306	0·038

analysis that will be readily applicable to most ester solutions, with or without excipients and irrespective of the presence of most other solutes.

From a comparison of the data in Tables 1 and 5 with the literature values also presented there, it is apparent that the present method gives results which are essentially in agreement

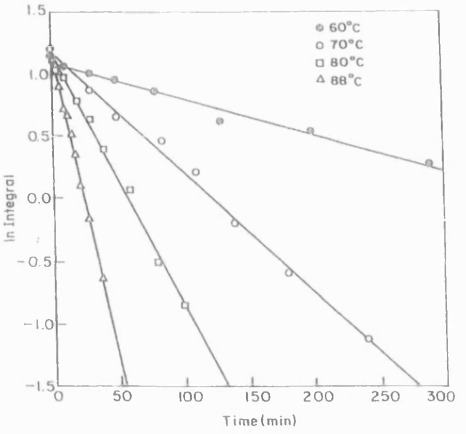

FIG. 1. Plot of ln integral of triplet at 5·06 ppm against time for atropine degradation at four different temperatures.

with those previously reported. The slightly higher values for first order rate constants for hydrolysis of procaine (Table 1) compared with Higuchi et al (1950) are probably a reflection of the higher concentration of buffer salts in the present study (0·25 M) compared with those used by Higuchi et al (1950) (0·05–0·2 M). The activation energies and frequency factors for the hydrolysis of acetylcholine, carbachol and atropine are in broad agreement with literature values (Table 5). The differences which are apparent are presumably a reflection of experimental protocol, such as temperature range, pH, buffer salts and drug concentration.

The inherent advantage of a spectroscopic over a chromatographic method of analysis, particularly where NMR is used, is that little method development is required once the general approach is established. In the present case a method was developed using procaine which was then applied, with little modification, to the other three esters. The procedure could equally well be applied to any other water-soluble ester, provided that a reporter group is present. This proviso may exclude esters of some tertiary alcohols which do not have a hydrogen atom in the sensitive position adjacent to the ester oxygen, although in these cases other spectral changes are likely which may allow the decomposition to be followed.

In principle any chemical degradation could be monitored by this method to obtain rate data, but where the peak to be monitored is further away from the water peak an alternative method for solvent suppression could be used, with some advantage in simplicity of sample preparation.

Table 5. Activation energies and frequency factors for hydrolysis of esters.

	Temperature (°C)	pH	Activation energy ($J\ mol^{-1}$)	Frequency factor (min^{-1})	Source
Acetylcholine	0–20	12·5–13	50 995	$1·00 \times 10^{9}$	Butterworth et al (1953)
	20–40	8·0–10·0	50 032	$1·47 \times 10^{9}$	Kunz (1973)
	45–65	7·0	53 549	$5·20 \times 10^{9}$	Present expt
Carbachol	70–90	0–7·7	93 784	$2·50 \times 10^{13}$	Lundgren (1969)
	70–90	8·5	97 163	$4·33 \times 10^{13}$	Present expt
Atropine	40–59·8	7·0–8·0	53 172	$2·10 \times 10^{10}$	Zvirblis et al (1956)
	60–88	8·0	47 596	$6·5 \times 10^{12}$	Present expt

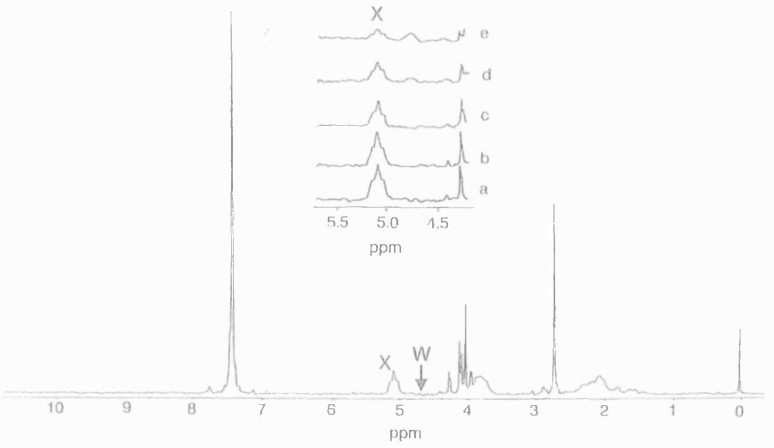

FIG. 2. Water-suppressed ^{1}H NMR spectrum of atropine before hydrolysis and (inserts) the peak at 5·06 ppm after (a) 0, (b) 5, (c) 20, (d) 40 and (e) 80 min. W marks the suppressed water peak, X marks the peak used to monitor the hydrolysis.

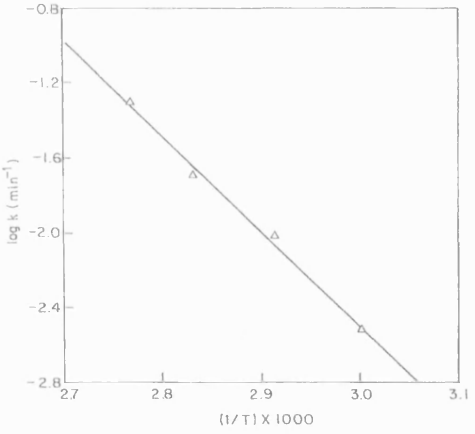

FIG. 3. Arrhenius plot of log k (min^{-1}) against reciprocal of absolute temperature of atropine hydrolysis.

We have shown previously that the optimum pH for water suppression varies with field strength (Dickinson et al 1987), as of course does T_2, reflecting the transverse relaxation rate which is significant in the WATR method. The machine used in this study is one of the least powerful allowing the use of the pulsed technique which is essential to the method. The use of machines operating at higher field would mean higher sensitivity, better signal to noise ratio and greater spectral resolution, all of which would improve the scope of the technique.

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References

Butterworth, J., Eley, D. D., Stone, G. S. (1953) Acetylcholine I. Hydrolysis by hydrogen and hydroxyl ion. Biochem. J. 53: 30–34

Dickinson, N. A., Lythgoe, R. E., Waigh, R. D. (1987) Some observations on the WATR method for water suppression at 80 MHz. Magn. Reson. Chem. 25: 996–997

Higuchi, T., Havinga, A., Busse, L. W. (1950) Kinetics of the hydrolysis of procaine. J. Am. Pharm. Assoc. Sci. Ed. 39: 405–410

Kunz, H. (1973) Untersuchung der alkalischen Hydrolyse von Oniumstern vom typ des Acetylcholins. Liebigs Ann. Chem. 2001–2009

Lundgren, P. (1969) Stability and stabilisation of carbachol in aqueous solutions. Acta Pharm. Suec. 6: 299–312

Rabenstein, D. L., Fan, S. (1986) Proton nuclear magnetic resonance spectroscopy of aqueous solutions: complete elimination of the water resonance by spin-spin relaxation. Anal. Chem. 58: 3178–3184

Rabenstein, D. L., Fan, S., Nakashima, T. T. (1985) Attenuation of the water resonance in Fourier transform ^{1}H NMR spectra of aqueous solutions by spin-spin relaxation. J. Magn. Reson. 64: 541–546

Zvirblis, P., Socholitsky, I., Kondritzer, A. A. (1956) The kinetics of the hydrolysis of atropine. J. Am. Pharm. Assoc. 45: 450–454

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Visualisation of a fluorinated pharmaceutical formulation in the anaesthetised rat using ^{19}F- and ^{1}H-NMR imaging

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Summary

The potential applicability of ^{19}F-NMR imaging in observing a fluorinated pharmaceutical in vivo using the rat as a model has been assessed. ^{19}F-NMR imaging was used in parallel with (^{1}H)-NMR imaging to observe and locate a solid dosage form in vivo. In vitro images of a tube phantom containing the fluorinated derivative were acquired initially in order to determine optimal acquisition parameters. Subsequently, in vivo ^{19}F images of the solid dosage form in the gastrointestinal tract of anaesthetised (fasted and nonfasted) rats were obtained and compared. Corresponding ^{1}H images were acquired at the mid-abdomen level of the rat. All images were acquired at a field strength of 4.7 T using a 16-segment copper birdcage resonator as the imaging probe. The ^{19}F images were obtained at the resonant frequency of 188.5 MHz and the proton images at the resonant frequency of 200.3 MHz using a spin-echo pulse sequence. Anatomical proton images showing gross structure of the gastrointestinal tract were acquired in as little as 2 min. Fluorine images were observed in approx. 3 min with a signal-to-noise (SNR) ratio of 7.5 per pixel.

Introduction

There has been an increasing awareness of the value in understanding the behaviour of pharmaceutical formulations in vivo (Anie et al., 1988). Transit times of orally administered pharmaceutical dosage forms through the gastrointestinal tract are of significance as drug absorption is considered to take place primarily from the small intestine. For this reason, the residence time of the dosage form in the stomach and the contact time with the absorption sites of the small intestine will influence the profile of drug absorption.

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The assessment of the localisation and movement of oral formulations has been examined previously using X-ray (Evans and Roberts, 1981) and radioisotope imaging including gamma scintigraphy (Davis et al., 1984; Fell and Digenis, 1984). To date, NMR imaging has not been used for this application. NMR imaging can, in theory, overcome problems inherent in the previous methods by permitting imaging of a much wider range of materials. Furthermore, ^{19}F-NMR imaging provides an as yet unexplored area for the localisation of formulations in the gastrointestinal tract. ^{19}F is a sensitive nucleus and is chosen for these investigations because of several specific advantages. These are the low intrinsic concentration of fluorine in soft tissue, the high NMR sensitivity of the fluorine nucleus, the 100% natural abundance of

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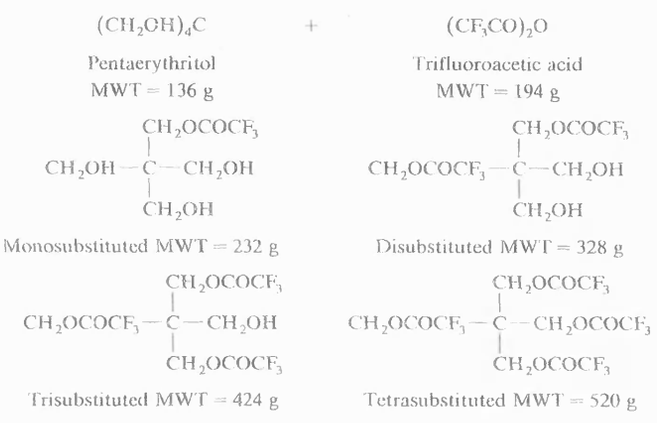

Scheme 1.

the ^{19}F isotope and the large gyromagnetic ratio of the fluorine nucleus, 25.17×10^7 rad T^{-1} s^{-1}, which is close to that of the proton nucleus, 26.75×10^7 rad T^{-1} s^{-1} thereby enabling the observation of ^{19}F with existing ^1H RF components.

Previous work on ^{19}F magnetic resonance spectroscopy and imaging has been largely concerned with various blood substitutes and fluorinated anaesthetics. The blood substitutes which have been studied include perfluorodecalin (Joseph et al., 1985), perfluorotributylamine (FTBA) (Longmaid et al., 1985), perfluorooctylbromide (PFOB) (Ratner et al., 1988) and perfluorophenanthrene (Thomas et al., 1986). There have also been investigations focused primarily on the potential applications of perfluorocarbons (PFCs) as contrast agents for a variety of organs (McFarland et al., 1985). Fluorinated anaesthetics such as halothane have been monitored using ^{19}F-NMR spectroscopy to detect the uptake and elimination from rabbit brain (Wyrwicz et al., 1983). A significant problem, however, in using fluorinated agents, especially at the high fields required for imaging, is the multiplicity of resonances arising from chemically non-equivalent fluorine atoms. These widely spaced resonances (50–150 ppm) introduce chemical shift artifacts in the frequency encoded spatial dimension.

For our investigations, this problem was overcome by synthesising pentaerythritol trifluoroacetates which are symmetrical structures with a single ^{19}F resonant frequency and using them in admixture.

Materials and Methods

Synthesis

Pentaerythritol (2 g) was refluxed with trifluoroacetic anhydride (8 ml) over an oil bath at 35 °C for 4 h (Scheme 1). The colourless liquid obtained was evaporated on a Buchi rotary evaporator to remove any trifluoroacetic acid and purified by a bulb-to-bulb distillation. Spectroscopic analyses was performed, infrared (IR) and NMR (both ^1H and ^{19}F). The product exhibited a predominant resonance line at 0.97 ppm with respect to pure trifluoroacetic acid used as a reference.

Animal studies

The animal studies utilised Sprague-Dawley male rats (200 g). Half the rats were in an 18 h fasted state and the other half had been fed normally. Each rat was anaesthetised (urethane, intraperitoneal; 5% in saline; 0.6 ml/100 g) prior to the oral administration of a pellet and imaging to

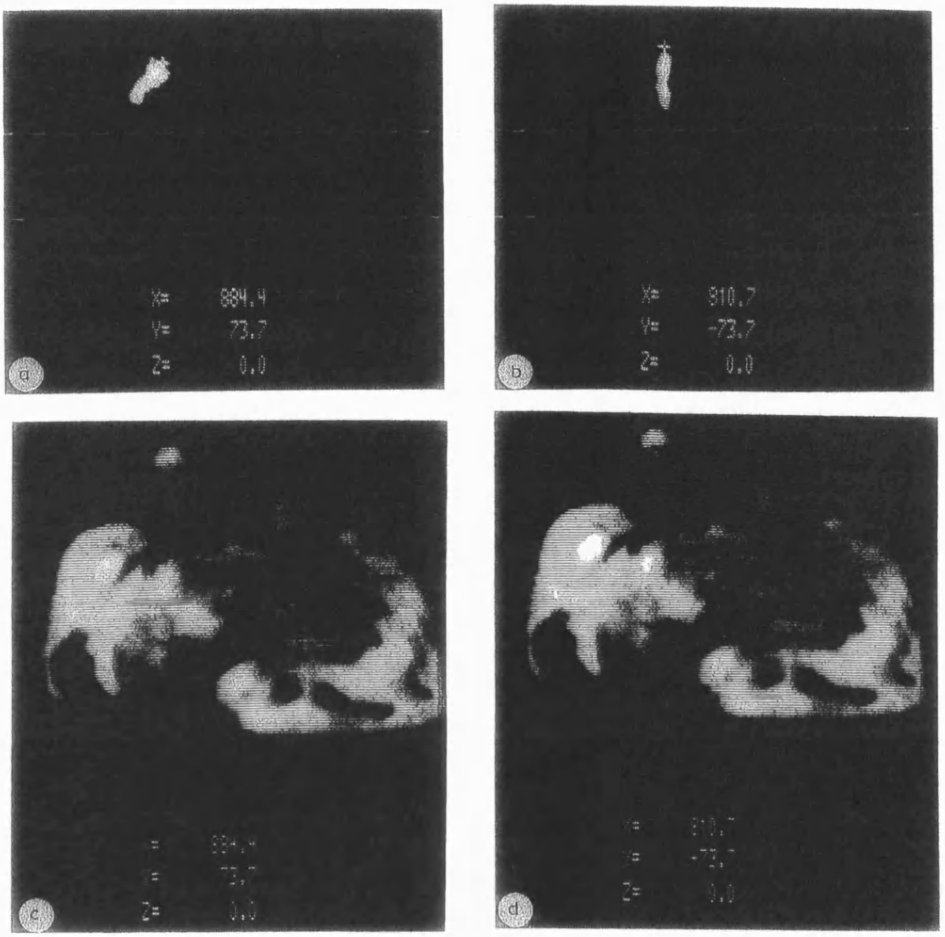

Fig. 1. (a, b) ^{19}F transverse images of a pellet in the gastrointestinal tract of a nonfasted rat. (a) 15 min after dosing; (b) 115 min after dosing. (c, d) Proton images corresponding to above after (c) 15 min and (d) 115 min after dosing.

enable immobilisation of the animal in the magnet. Hollow capsule-shaped pellets made of Nylon-6,6 (bore 2.2 mm, diameter 2.6 mm, length 7.0 mm) were filled with the fluorinated formulation (0.006 g) and sealed to prevent leakage. An administration tube comprising a Luer stainless-steel needle and cone hollowed out to form a cup for the pellet was used during oral dosing of the rat. The pellet was deposited into the distal end of the oesophagus. This procedure was duplicated for

all the studies. The rat was then placed supine in a perspex restraint cage and positioned in the birdcage coil. Each rat was killed (halothane inhalation) and dissected after the experiment to ascertain the position of the pellet.

Instrumentation / imaging protocol

In vivo images were acquired on an ORS/Bruker biospectrometer (Oxford Research Systems, Abingdon, U.K) operating at a field strength of 4.7 T. A 16-segment copper birdcage resonator was employed as the imaging probe with corresponding 90 and 180° pulse lengths of 190 and 380 μs respectively. The ^{19}F images were obtained at the resonant frequency of 188.5 MHz and the proton images at the resonant frequency of 200.3 MHz. A two-dimensional FT sequence was used to obtain all images. All images consisted of 128 × 128 independent picture points. Image acquisition was commenced 15 min after the administration of the pellet to the rat. The animals underwent both ^{19}F and ^{1}H scanning in the birdcage coil tuned successively from the ^{19}F frequency to the ^{1}H frequency. There was a time lag of approx. 2 min during which the birdcage was retuned and rematched. ^{19}F imaging was used to locate the pellet in vivo and ^{1}H imaging to assign the pellet to a specific region of the rat. Transverse images were acquired at the level of the abdomen at time intervals to note gross movement of the pellet.

The pulse repetition time for both ^{19}F and ^{1}H imaging, TR, was 1045.2 ms and the echo time, TE, was 35.6 ms. The slice thickness of each image was 4 mm. The proton scans took 2 min to acquire. Due to the low signal-to-noise ratio of the ^{19}F resonance, image acquisition of the fluorine images took approx. 3 min.

Results and Discussion

Fig. 1a and b shows ^{19}F transverse images of a pellet in the gastrointestinal tract of a nonfasted rat, acquired 15 and 115 min, respectively, after dosing. The cursor position shown by the coordinates indicates the position of the pellet, and is different in Fig. 1a as compared with Fig. 1b. The pellet appears to be changing in position and is moving in the anticlockwise direction. Proton images corresponding to the ^{19}F images acquired 15 and 115 min after dosing are shown in Fig. 1c and d, respectively. These depict structures such as the spinal canal, muscle, stomach, kidneys, small and large intestines, bowel gas and body wall. The cursor positions on the proton images mark the location of the pellet with time. For the proton images, the cursor indicates the pellet is in the stomach of the rat and remains in the stomach even after 265 min. On account of the varying cursor coordinates on the ^{19}F images, one may conclude that the pellet is moving in vivo. The minor changes in pellet position could be attributed to the fact that the experiment was performed with a nonfasted rat, therefore the stomach contained ingested food before the experiment was commenced. The pellet mixes with food in the stomach and its movement is restricted. Furthermore, the rat was anaesthetised prior to imaging and it is likely that urethane interferes with the normal peristaltic waves of the stomach thus inhibiting gastric emptying of the pellet. On dissection, the pellet was located in the forestomach close to the limiting ridge and imbedded in food remnants within the stomach of the rat.

Fig. 2a and b are ^{19}F transverse images of a pellet in vivo acquired with a fasted rat. The movement of the pellet as indicated by the cursor coordinates appears to be more pronounced in this case as compared with the nonfasted state. In Fig. 2a, acquired 15 min after dosing, the fluorine image of the pellet appears vertical. However, 265 min after dosing (see Fig. 2b) there appears to be an anticlockwise tilt of the pellet from its previous position. Selected proton images corresponding to fluorine images obtained 15 and 265 min after dosing are provided in Fig. 2c and d, respectively. The proton scans differ and reveal that the pellet has moved significantly during the experiment as compared with the results obtained with the nonfasted state. On dissection, the pellet was located in the pylorus at the junction of the duodenum. In the absence of urethane, used as an anaesthetic to immobilise the animals during these experiments, motility of the pellet could be enhanced resulting in the observation of the pellet in the intestinal processes of the rat. However, it is essential that

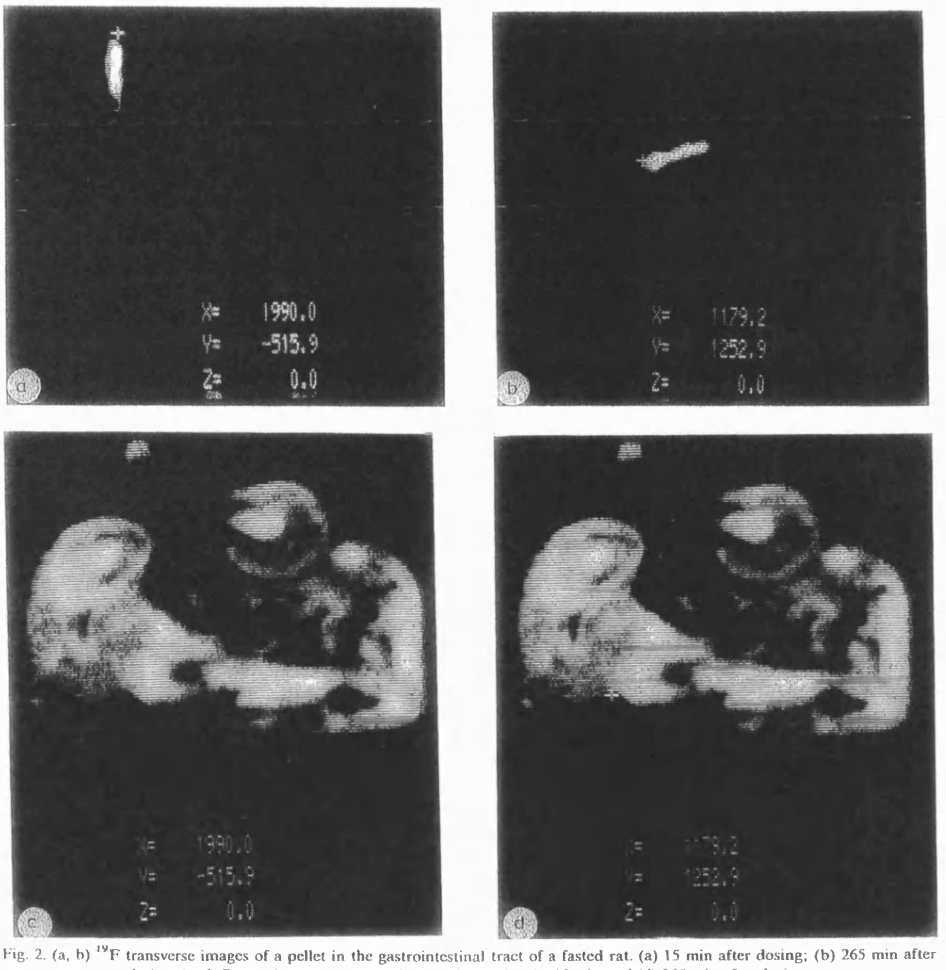

Fig. 2. (a, b) ^{19}F transverse images of a pellet in the gastrointestinal tract of a fasted rat. (a) 15 min after dosing; (b) 265 min after dosing. (c, d) Proton images corresponding to above after (c) 15 min and (d) 265 min after dosing.

movements of the animal are prevented as far as possible to improve image quality and to reduce motional artifacts on the image, which are already prevalent due to normal heart function and respiratory motion of the animal. Perhaps the use of a sedative such as diazepam would alleviate this problem. Overall greater movement of the pellet occurs in the fasted state as deduced from these experiments using ^{19}F- and ^{1}H-NMR imaging.

These images represent initial results on the use

of ^{19}F- and ^{1}H-NMR imaging to observe and localise a solid dosage form in the gastrointestinal tract. In these studies advantage is taken of the fact that there are no background signals, originating from the animal, so that signals observed are exclusively from the pellet containing the fluorinated derivative. The initial aim was to select a product containing a high proportional mass of fluorine and a single line in its NMR spectrum, with convenient handling characteristics. Another important feature of these studies was the accurate spatial correlation that was achieved between the fluorine and proton images. This was possible due to the selection of the birdcage resonator as the imaging probe. The birdcage resonator may be tuned and matched to the appropriate frequency between successive ^{19}F and ^{1}H imaging without moving the animal or resonator because the respective resonant frequencies of ^{19}F and ^{1}H are close, i.e 188.5 and 200.3 MHz, respectively. The reliability of the administration procedure means that provided the administration tube reaches the distal end of the oesophagus, pellets deposited reach the stomach within 10 min.

The relative sensitivity of fluorine is 94% to that of hydrogen at constant frequency and therefore, fluorine and hydrogen produce similar signal strengths as observed in our images. The acquisition time associated with the ^{19}F images, 3 min, shows an improvement on previously published data. The geometry of the birdcage imaging probe is such that the restraint cage containing the rat fills the volume of the probe, so that detection sensitivity is maximised and a better SNR is realised.

Further studies would involve the use of non-anaesthetised rats or sedated rats to enable greater movement of the pellet in vivo. Other ^{19}F imaging agents could be formulated having the characteristics previously mentioned. These could be potentially administered in dosage forms and the entire movement through the GI tract observed. We conclude that the method is sufficiently fast and sensitive to obtain data regarding transit times.

References

Anie, S.J., Fell, J.T. and Waigh, R.D., Magnetic resonance imaging for the detection and localisation of a pharmaceutical formulation in vivo. *J. Pharm. Pharmacol.*, 40 (1988) 128P.

Davis, S.S., Hardy, J.G., Taylor, M.J., Whalley, D.R and Wilson, C.G., A comparative study of the gastrointestinal transit of a pellet and tablet formulation. *Int. J. Pharm.*, 21 (1984) 167–177.

Evans, K.T. and Roberts, G.M., The ability of patients to swallow capsules. *J. Clin. Hosp. Pharm.*, 6 (1981) 207–208.

Fell, J.T. and Digenis, G.A., Imaging and behaviour of solid dosage forms in vivo. *Int. J. Pharm.*, 22 (1984) 1–15.

Joseph, P.M., Fishman, J.E., Mukherji, B. and Sloviter, H.A., In vivo ^{19}F-NMR imaging of the cardiovascular system. *J. Comput. Assist. Tomogr.*, 9 (1985) 1012–1019.

Longmaid III, H.E., Adams, D.F., Neirinck, R.D., Harrison, C.G., Brunner, P., Seltzer, S.E., Davis, M.A., Neuringer, L. and Geyer, R.P. In vivo ^{19}F-NMR imaging of liver, tumor and abscess in rats. *Invest. Radiol.*, 20 (1985) 141–145.

McFarland, E., Koutcher, J.A., Rosen, B.R., Teicher, B. and Brady, T.J., In vivo ^{19}F-NMR imaging. *J. Comput. Assist. Tomogr.*, 9 (1985) 8–15.

Ratner, A.V., Hurd, R., Muller, H.H., Bradley-Simpson, B., Pitts, W., Shibata, D., Sotak, C. and Young, S.W., ^{19}F magnetic resonance imaging of the reticuloendothelial system. *Magn. Reson. Med.*, 5 (1988) 548–554.

Thomas, S.R., Clark Jr, L.C., Ackerman, J.L., Pratt, R.G., Hoffmann, R.E., Busse, L.J., Kinsey, R.A., and Samaratunga, R.C., NMR imaging of the lung using liquid perfluorocarbons. *J. Comput. Assist. Tomogr.*, 10 (1986) 1–9.

Wyrwicz, A.M., Pszenny, M.H., Schofield, J.C., Tillman, P.C., Gordon, R.E. and Martin, P.A., Noninvasive observations of fluorinated anaesthetics in rabbit brain by fluorine 19 nuclear magnetic resonance. *Science*, 222 (1983) 428–430.

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Notes

Gastrointestinal transit studies in the rat using ^{19}F-NMR

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Summary

^{19}F-NMR has been used to assess the movement of a non-disintegrating pellet containing a fluorinated derivative of pentaerythritol in the gastrointestinal tract of a rat. The data were treated using computer graphics to provide a three-dimensional representation of the movement of the pellet.

The behaviour and transit of solid oral dosage forms in the gastrointestinal tract are related to drug absorption. NMR imaging is a potentially valuable method of obtaining such data and may have advantages over gamma scintigraphy, the current method of choice.

Previous studies have shown that ^{19}F-NMR imaging may be used in conjunction with ^{1}H-NMR imaging to observe and locate a pellet in vivo (Anie et al., 1989; Anie, 1990). A significant problem encountered is the time associated with the acquisition of the images. Due to the rapid movement of the pellet in the gastrointestinal tract of the rat, a faster method of assessment would be useful. In view of this, ^{19}F-NMR has been investigated for this application without the requirement for the generation of images.

Studies utilised 200 g sedated (diazepam; intraperitoneal; 2.5 mg/kg) or anaesthetised (urethane; intraperitoneal; 5% in saline; 0.6 ml/100 g) Sprague-Dawley male rats, in an 18 h starved state. Hollow capsule-shaped pellets were filled with a fluorinated derivative of pentaerythritol (0.006 g) (Anie et al., 1991) and sealed to prevent leakage. A single pellet was orally admin-

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istered into the stomach. A reference pellet containing the derivative was fixed to the dorsal region of the rat. The sedated or anaesthetised rat was placed in a perspex restraint cage, positioned in a birdcage coil and finally into the magnet. Each rat was killed and dissected on completion of the study to ascertain the position of the pellet in the gastrointestinal tract and to correlate this position with the NMR data.

Data acquisition was commenced 15 min after the administration of the pellet to the rat. The instrument used was an ORS/Bruker biospectrometer. The experiment was performed at 4.7 T with the birdcage coil tuned and matched to 188 MHz. Pulses of 90 and 180° were applied for 275 and 550 μs, respectively. The sequence repetition time (TR) was 2031 ms and the excitation pulse to echo duration (TE) was 24.8 ms. A one-dimensional experiment in each of the three gradients x, y and z allows rapid location of the centre of the pellets with a precision of roughly 0.5 mm. Field gradients used in the x, y and z directions were 2558, 2408 and 2257 Hz/cm, respectively. To increase the signal-to-noise ratio, each signal was acquired repeatedly, i.e. averaged. Signal averages were obtained approximately every 2 min for 5 h.

In each experiment, ^{19}F-NMR signals originating from the two pellets were obtained. Measurements made relative to the reference pellet (R) provided information as to the pellet mobility. Fig. 1 shows a selected portion of the Z profile acquired from a sedated rat. Each field map consists of 22 ^{19}F spectra and shows two peaks, one corresponding to the reference and the other to the pellet in the gastrointestinal tract. A three-dimensional pattern of movement relative to the reference pellet can thus be obtained. A greater movement of the pellet was obtained when the rats were sedated as opposed to being anaesthetised by urethane. This was confirmed on dissection.

The data can be treated using computer graphics to give a visual representation of the pellet movement. Typical displays are shown in Fig. 2a and b for anaesthetised and sedated rats depicting greater movement after sedation. The x, y and z co-ordinates are displayed to show the viewing plane. The plots can be rotated to obtain the three-dimensional image.

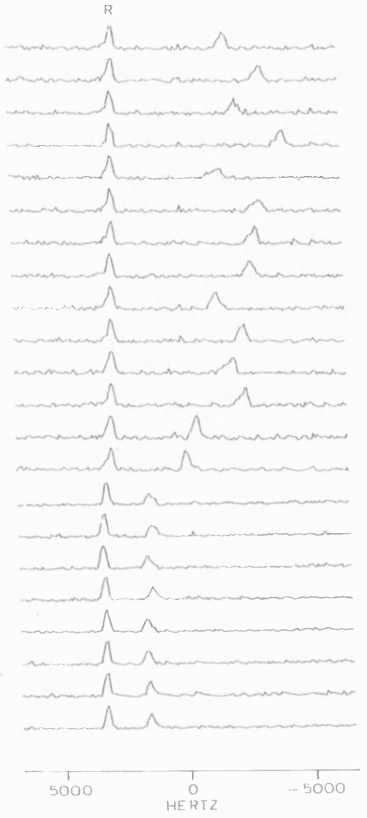

Fig. 1. The Z profile of a pellet in a sedated rat (scale: 1000 units = 0.44 cm).

These studies coupled with previous work (Anie et al., 1991) demonstrate the non-invasive feature and potential of NMR methods for gastrointestinal transit studies. Linking NMR spec-

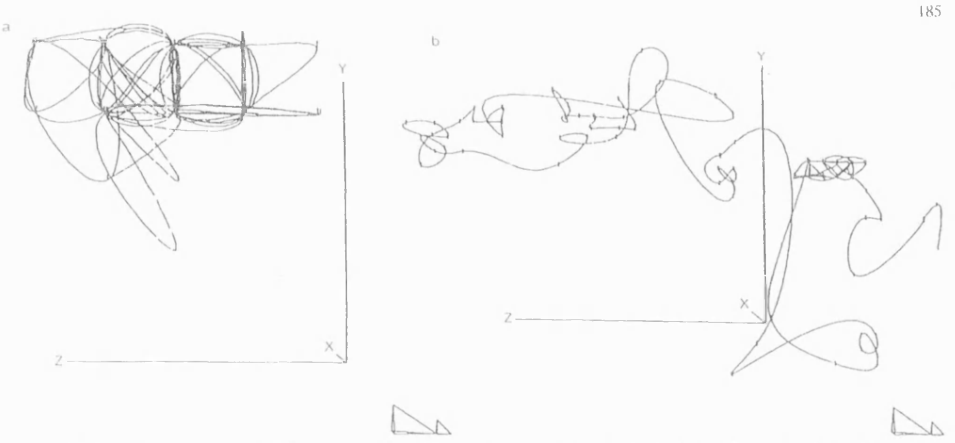

Fig. 2. (a) A three-dimensional plot of the pathway of a pellet acquired with an anaesthetised rat. (b) A three-dimensional plot of the pathway of a pellet acquired with a sedated rat.

troscopy with computer graphics allows rapid data acquisition and a visual representation of transit.

References

Anie, S.J., ^{19}F- ^{1}H-NMR studies of materials in the gastrointestinal tract, PhD Thesis, University of Manchester (1990).

Anie, S.J., Fell, J.T., Waigh, R.D. and Wood, B., The use of fluorine-19 NMR to monitor a pharmaceutical formulation 'in vivo'. *Bull. Magn. Reson.*, 11 (1989) 412.

Anie, S.J., Fell, J.T. and Waigh, R.D., Visualisation of a fluorinated pharmaceutical formulation in the anaesthetised rat using ^{19}F and ^{1}H-NMR imaging. *Int. J. Pharm.*, 69 (1991) 181–186.

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Application of the WATR Technique for Water Suppression in ^{1}H NMR Spectroscopy in Determination of The Kinetics of Hydrolysis of Neostigmine Bromide in Aqueous Solution

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Abstract—Both ammonium chloride and guanidinium chloride were used to secure water suppression in ^{1}H NMR spectra using the 'Water Attenuation by T_2 Relaxation' (WATR) technique. The effect of phosphate buffer in the suppression was investigated over a range of pH values at 80 MHz. The spin–spin relaxation time of water protons at 80 MHz was found to reach a minimum at pH 7·3 in the presence of 0·1 M phosphate buffer and 1 M guanidinium chloride; these conditions were therefore chosen for subsequent use of the WATR technique in a study of the kinetics of hydrolysis of neostigmine bromide. The method was found to be very convenient for studies of the hydrolysis of this representative amide.

Nuclear magnetic resonance (NMR) spectroscopy has been widely used in the study of drug degradation rates (Mitsumori et al 1977; Baltzer et al 1979; Degelaen et al 1979; Nishikawa et al 1987). As an analytical tool it offers various advantages compared with other methods of analysis (Ferdous 1989). Drugs undergoing hydrolytic degradation in aqueous solution are not amenable to direct ^{1}H NMR spectroscopic analysis; the high concentration of water protons (ca. 110 M) causes dynamic range problems in recording the NMR spectra and the huge water peak obscures or distorts all nearby weak solute peaks. The problem of removing the water peak from the spectra of aqueous solutions has been successfully overcome by using the 'Water Attenuation by T_2 Relaxation' (WATR) method (Rabenstein et al 1985). Using this method, peaks in the region of δ 4–5 ppm can be easily observed (Rabenstein & Fan 1986; Dickinson et al 1987). Various relaxation agents have been used to suppress the water peak. In the present study the relaxation effect of ammonium chloride and guanidine hydrochloride and the effect of pH on water suppression are reported. As an extension of the use of the technique in studies of ester hydrolysis (Ferdous et al 1991), the method has been used to study the hydrolysis rate of a representative amide, neostigmine bromide.

Materials and Methods

Ammonium chloride (BDH, Poole, Dorset), guanidine hydrochloride (Aldrich, Gillingham), acetamide (BDH), potassium dihydrogen phosphate and sodium hydroxide were of analytical grade. Deuterium oxide (99·88%D) was obtained from Fluorochem (Glossop). Neostigmine bromide (Aldrich) was used without further purification. Distilled water was used in all experiments.

The spin–spin relaxation time (T_2) was measured using the CPMG pulse sequence (Carr & Purcell 1954; Meiboom & Gill 1958). The sample solution contained 5% (v/v) D_2O and a measured amount of relaxation agent (ammonium chloride or guanidine hydrochloride). The solution contained buffer salts to stabilize pH. About 0·5 mL solution was used in a 5-mm diameter NMR tube. The CPMG data were obtained as a stack plot. Digital data printouts were used to calculate T_2 by least square regression analysis.

The water suppressed ^{1}H NMR spectra of neostigmine bromide samples in aqueous solution were recorded using the WATR method (Ferdous et al 1991). The experiments were carried out on a Bruker WP80 NMR spectrometer operating at 80 MHz at 20°C.

The degradation of neostigmine bromide was studied at four different temperatures at pH 9·3. Water (50 mL) was adjusted to the desired pH. A flask containing 45 mL of this solution was placed in the water bath and kept there until it reached the required temperature. Neostigmine bromide (1% w/v) was measured and dissolved in the remaining 5 mL solution and the pH was adjusted. The two solutions were mixed and transferred to a jacketed vessel through which hot water was circulated to maintain the desired temperature. The jacketed vessel was placed in a pH-stat and the pH was constantly monitored. As the degradation continued the pH of the solution was kept constant by adding sodium hydroxide solution (0·7–1·4 M) automatically. Samples were collected at the beginning and at timed intervals. Each sample was placed in a screw-cap tube, instantly frozen in liquid nitrogen and kept at −6°C until the spectrum was recorded. Before obtaining the spectrum, the sample was thawed and a solution was added containing acetamide (0·075% w/v) as internal standard, 0·1 M phosphate buffer, 5% (v/v) D_2O for an NMR lock signal and 1 M guanidine hydrochloride as relaxation agent. The final pH of the solution was adjusted to 7·3. A total of 32 scans was accumulated for each spectrum with a total delay of 0·8 s.

After acquisition of NMR spectra, digital printouts were obtained containing peak area (integral) for each chemical shift. The integral of the two singlets at δ 3·1 ppm was normalized with reference to the integral of the internal standard acetamide peak at δ1·9 ppm. Three spectra were recorded for each sample. The integrals for the peak from

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each spectrum after normalization were added together and the average value was taken. The ln integral values were plotted against time for each degradation experiment. The slope of the line of best fit was computed which gave the first order rate constant for that degradation experiment. All experiments were performed in triplicate and the average value was recorded.

Results and Discussion

The spin–spin relaxation time (T_2) of water protons was determined in varying concentrations of phosphate buffer and at two different pH values. The change in T_2 of water protons in the presence of 0·25 M ammonium chloride as relaxation agent and increasing concentration of phosphate buffer at pH 5·5 and 6·0 is shown in Fig. 1. The change in T_2 of water protons with increasing concentration of phosphate buffer in the presence of 1 M ammonium chloride at pH 5·5 is shown in Fig. 2. For Fig. 1, two pH values were chosen because the relaxation effect of ammonium chloride was maximum around the pH range 5·5–6·0 (Dickinson et al 1987). The T_2 of water protons increased with increasing phosphate buffer up to a maximum (0·35–0·45 M; Figs 1, 2); T_2 then slightly decreased with further increase of phosphate buffer. The spin–spin relaxation times are slightly longer at pH 6·0 than at pH 5·5 in the presence of the same

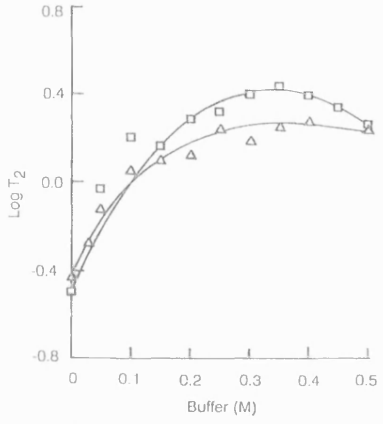

FIG. 1. Ln T_2 of water protons (in presence of 0·25 M ammonium chloride at pH 5·5 (△) and 6·0 (□)) plotted as a function of phosphate-buffer concentration.

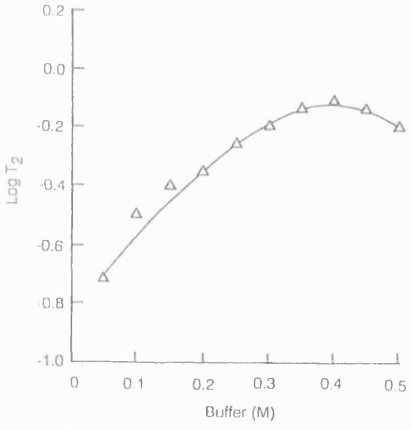

FIG. 2. Ln T_2 of water protons (in presence of 1 M ammonium chloride at pH 5·5) plotted as a function of phosphate-buffer concentration.

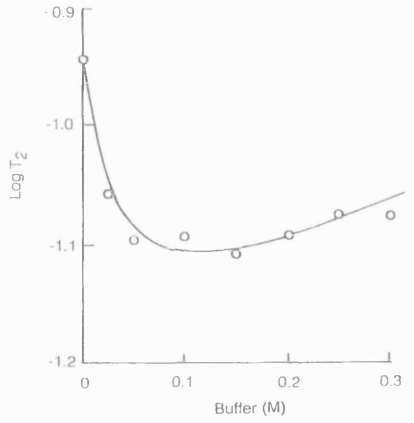

FIG. 3. Ln of spin–spin relaxation time (T_2) of water protons (in presence of 1 M guanidine hydrochloride at pH 7·2) plotted as a function of phosphate-buffer concentration.

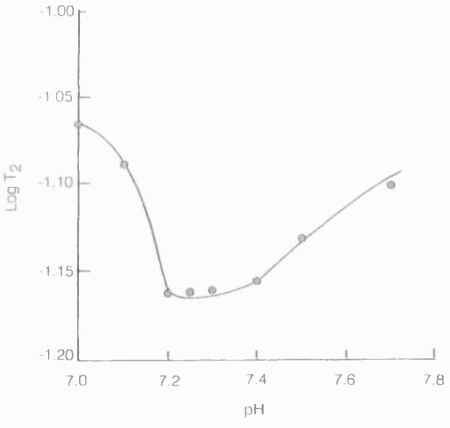

FIG. 4. pH dependence of Log T_2 of water protons in the presence of 1 M guanidine hydrochloride and 0·1 M phosphate buffer.

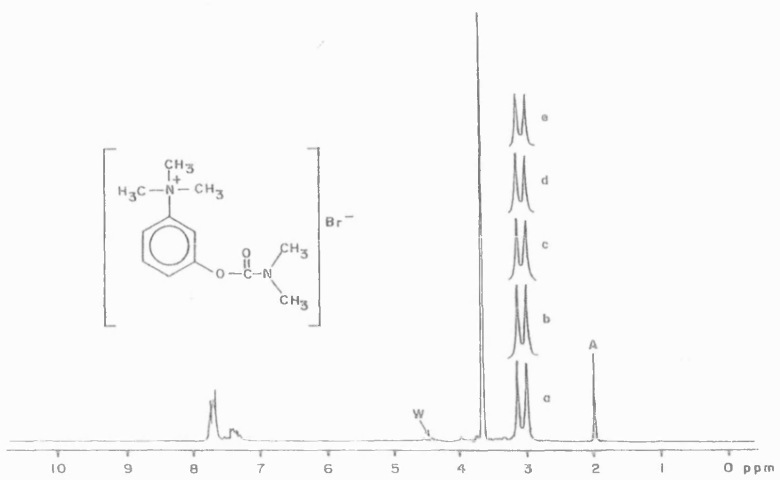

FIG. 5. Water suppressed ^{1}H NMR spectrum of neostigmine bromide before hydrolysis and (inserts) the double singlets at δ 3·1 ppm after (a) 0, (b) 30, (c) 60, (d) 120 and (e) 180 min. Samples were degraded at 70°C. W marks the suppressed water peak, and A acetamide peak. The structure of neostigmine bromide is given in the insert.

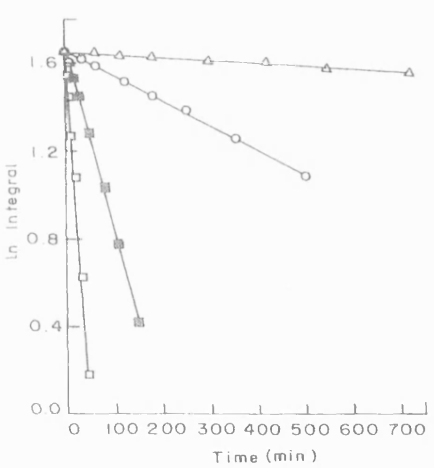

FIG. 6. Plot of ln integral of double singlet at δ 3·1 ppm against time for neostigmine bromide degradation at four different temperatures and at pH 9·3. △ 60, ○ 70, ▨ 80 and □ 88°C.

Table 1. First order rate constants for hydrolysis of neostigmine bromide at pH 9·3.

Temperature (°C)	K (min^{-1})	Log K	s.d.
60	$1{\cdot}38947 \times 10^{-4}$	−3·857144	0·01134
70	$1{\cdot}13133 \times 10^{-3}$	−2·946410	0·01215
80	$8{\cdot}39509 \times 10^{-3}$	−2·075974	0·01956
88	$3{\cdot}83084 \times 10^{-2}$	−1·416706	0·01057

Table 2. Activation energy for hydrolysis of neostigmine bromide.

Temperature (°C)	pH	Activation energy (kJ mol^{-1})	Source
70–90	7·6	101·1	Porst & Kny (1985)
10–45	13·0	14·0*	Christenson (1964)
60–88	9·3	43·9	Present study

* Calculated from Christenson (1964).

concentration of phosphate buffer and ammonium chloride. Increasing the ammonium chloride concentration from 0·25 to 1 M significantly decreased the relaxation time (Fig. 2) which is in accordance with previous findings (Dickinson et al 1987). Phosphate buffer at pH 5·5 is slightly below its recommended buffering range. Phosphate buffers at or above pH 5·5 can be used in obtaining the WATR spectra, but a higher concentration of ammonium chloride would be necessary at higher pH values for complete suppression of the water signal.

The effect of phosphate buffers on T_2 of water protons in the presence of 1 M guanidine hydrochloride is shown in Fig. 3. T_2 decreased with increase of phosphate buffer up to 0·05 M. In the range of 0·05–0·2 M phosphate buffer, T_2 was almost constant. Above that concentration, there was a slight increase in the T_2 of the water protons. Therefore, subsequently the WATR spectra of all compounds were measured in 0·1 M phosphate buffer. The relaxation effect of guanidine hydrochloride was greater than that of ammonium chloride (Dickinson et al 1987). Guanidine hydrochloride was also found to be less susceptible to interference from phosphate

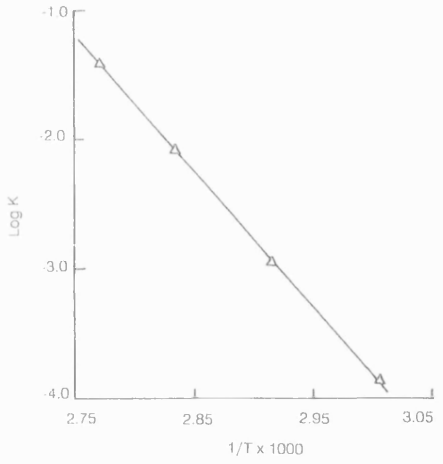

FIG. 7. Arrhenius plot of ln K (min^{-1}) against reciprocal of absolute temperature of neostigmine hydrolysis degradation.

buffers (Fig. 3), so guanidine hydrochloride was used as the relaxation agent to suppress the water peak in all subsequent experiments.

The T_2 of water protons in 0·1 M phosphate buffer and 1 M guanidine hydrochloride in the pH range 7·0–7·7 was determined: the results are presented in Fig. 4. In this pH range in unbuffered solution Dickinson et al (1987) found T_2 to be at a minimum. In phosphate buffer, the T_2 was found to be at a minimum in the pH range 7·2–7·4. Above and below the pH range, T_2 increased significantly. Therefore, pH 7·3 was chosen as the optimum pH for obtaining WATR spectra in the presence of 0·1 M phosphate buffer and 1 M guanidine hydrochloride as the relaxation agent.

The hydrolysis rate of neostigmine bromide was determined at 60, 70, 80 and 88°C at pH 9·3. The WATR spectrum was recorded for each sample obtained during each degradation experiment. Since spectra were obtained at pH 7·3, at which pH hydrolysis is likely to be slow (compare data in Table 2) and at ambient temperature, ca. 20°C (compare data in Fig. 6), it can be assumed that hydrolysis during acquisition of the WATR spectra would be negligible. A typical spectrum of neostigmine bromide at the beginning of degradation is shown in Fig. 5. The integral of the two methyl singlets at δ 3·1 ppm thus obtained was normalized with the integral of the peak of the internal standard acetamide. The amide methyl peaks at δ 3·1 ppm were chosen because they were well separated from the peak for dimethylamine at ca. δ 2·7, present after hydrolysis. The ln integral (after normalization) was then plotted against time (Fig. 6). The slope of each line gave the rate constant (K) for hydrolysis (Table 1). The Arrhenius plot (Fig. 7) was obtained by plotting the rate constant for hydrolysis against the reciprocal of absolute temperature. From the slope of the line, the activation energy was calculated. The results thus obtained can be compared (Table 2) with those reported by Christensen (1964) and Porst & Kny (1985). The differences in Table 2 are due to temperature, pH, buffer salts and drug concentration. At low pH, more energy is required for hydrolysis, so the activation energy is high; at higher pH values the activation energy is lower.

The WATR technique has the advantage over chromatographic procedures in that there is little method development, and separation of degraded products from the parent compound is not necessary. The method is rapid and straightforward and both undegraded and degraded products can be analysed and quantified easily. In this experiment an NMR spectrometer of relatively low sensitivity and resolving power was used. If an instrument of higher field strength were used then greater spectral resolution and better signal-to-noise ratio would be obtained which would further improve the scope of this technique.

Acknowledgements

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References

Baltzer, B., Lund, F., Rustrup-Anderson, N. (1979) Degradation of mecillinam in aqueous solution. J. Pharm. Sci. 68: 1207–1215

Carr, H. Y., Purcell, E. M. (1954) Effects of diffusion on free precession in nuclear magnetic resonance experiments. Phys. Rev. 94: 630–639

Christenson, I. (1964) Alkaline hydrolysis of some carbamic acid esters. Acta Chem. Scand. 18: 904–922

Degelaen, J. P., Loukas, S. L., Feeney, J., Roberts, G. C. K., Burgen, A. S. V. (1979) A nuclear magnetic resonance study of degradation of Penicillin-G in acidic solution. J. Chem. Soc. Perkin Trans. II: 86–90

Dickinson, N. A., Lythgoe, R. E., Waigh, R. D. (1987) Some observations on the WATR method for water suppression at 80 MHz. Magn. Reson. Chem. 25: 996–997

Ferdous, A. J. (1989) NMR Spectroscopy in the Study of Drug Degradation Rates. Ph D Thesis, University of Manchester, UK

Ferdous, A. J., Dickinson, N. A., Waigh, R. D. (1991) ^{1}H NMR as an analytical tool for the investigation of hydrolysis rates: a method for rapid evaluation of the shelf-life of aqueous solutions of drugs with ester groups. J. Pharm. Pharmacol. 43: 860–862

Meiboom, S., Gill, D. (1958) Modified spin-echo method for measuring nuclear relaxation times. Rev. Sci. Instrum. 29: 688–691

Mitsumori, F., Arata, Y., Fujiwara, S., Muranaka, M. (1977) NMR study of spontaneous degradation of penicillin G in aqueous solution. Bull. Chem. Soc. Japan 50: 3164–3166

Nishikawa, J., Watanabe, F., Shudou, M., Terui, Y., Narisada, M. (1987) ^{1}H NMR study of degradation mechanisms of oxacepham derivatives with various 3′-substituents in alkaline solution J. Med. Chem. 30: 523–527

Porst, H., Kny, L. (1985) Zur kinetic des neostigminabbaus in wasringer losung. Pharmazie 40: 713–717

Rabenstein, D. L., Fan, S. (1986) Proton nuclear magnetic resonance spectroscopy of aqueous solutions: complete elimination of the water resonance by spin-spin relaxation. Anal. Chem. 58: 3178–3184

Rabenstein, D. L., Fan, S., Nakashima, T. T. (1985) Attenuation of the water resonance in Fourier transform ^{1}H NMR spectra of aqueous solutions by spin–spin relaxation. J. Magn. Reson. 64: 541–546

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^{1}H and ^{13}C NMR Studies of the Self-Association of Chlorpromazine Hydrochloride in Aqueous Solution

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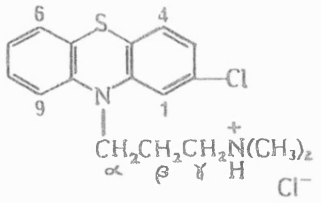
The ^{1}H NMR spectrum of chlorpromazine hydrochloride was fully assigned at 400 MHz. Similarly, the ^{13}C NMR spectrum was assigned unambiguously using two-dimensional NMR. Measurements of chemical shift as a function of concentration in D_2O showed appreciable changes of shift of both protons and carbons which were apparent even at solution concentrations two orders of magnitude lower than the critical micelle concentration (CMC). The relative magnitude of the shifts of the aromatic protons and carbons on dilution below the CMC were compatible with vertical stacking of the molecules in an off-set manner such that maximum overlap of the chlorinated rings occurred. Proton chemical shift data were interpreted using a stepwise association model to quantify the extent of association in the pre-CMC region.

KEY WORDS Chlorpromazine ^{1}H NMR ^{13}C NMR Association models

INTRODUCTION

The pharmacological groups of tranquillizing drugs based on the phenothiazine ring system are surface active and exhibit self-association in aqueous solution. The occurrence of an abrupt change in physicochemical properties at a critical concentration led early workers[1] to assume a micellar mode of association, the inflection being identified with the critical micelle concentration (CMC) of typical surfactants. More recent work has cast doubt on this inference. The demonstration of additional discontinuities in the light-scattering data of several phenothiazine drugs at higher solution concentration[2,3] suggests a more complex association pattern for these drugs. There is also recent evidence from calorimetric,[4–6] osmotic,[7] e.m.f. and ultrasonic relaxation[8] studies that limited association occurs below the first critical concentration in water and dilute electrolyte. It is this aspect of the association process that was addressed in this study.

An earlier study[9] of the changes in the proton chemical shifts of the $^{+}NH(CH_3)_2$ group, the $CHCH_3$ and the aromatic region of the phenothiazine molecule on dilution led to the suggestion of vertical stacking of these drugs in the manner of the tricyclic dyes. In this paper we report a more detailed examination of the changes in chemical shift, both ^{1}H and ^{13}C, over a wide concentration range. The use of a much higher field strength than previous workers who have studied the phenothiazine drugs[9–16] allowed the observation of changes in chemical shift of individual aromatic protons and carbon atoms of chlorpromazine (1) extending to regions of high dilution, from which deductions of the orientation of the molecules in the stacks have been made.



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EXPERIMENTAL

Chlorpromazine hydrochloride (Sigma Chemical) conformed to the purity requirements of the British Pharmacopoeia and, as such, contained not less than 98.5%

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Table 1. H,H coupling constants for chlorpromazine hydrochloride, 11.12 mmol kg^{-1} in D_2O[a]

	Coupling constant (Hz)
$^3J(\alpha\beta) = {}^3J(\alpha\beta') = {}^3J(\alpha'\beta) = {}^3J(\alpha'\beta')$	6.5
$^3J(\beta\gamma) = {}^3J(\beta'\gamma)$	10.6
$^3J(\beta\gamma) = {}^3J(\beta'\gamma)$	5.6
$^4J(13)$	2.0
$^3J(34)$	8.2
$^3J(67) = {}^3J(78)$	7.6
$^4J(68)$	1.5
$^4J(79)$	1.1
$^3J(89)$	8.1

[a] Values for the protons for the side-chain were calculated using the simulation program LAOCOON; those for the ring protons are experimental values.

of the specified compound. All solutions were prepared by mass in 99.93% deuterium oxide immediately prior to use to minimise photodegradation. Errors arising from adsorption of the surface-active drug on to glassware in very dilute solution were minimized by soaking the glassware with solutions of identical concentration for approximately 2 h, thus saturating the walls of the containers, before replacing with fresh solution immediately before measurement. Any storage of solutions was in the dark to reduce photodegradation.

^1H and ^{13}C NMR spectra were assigned using a Bruker AM400X spectrometer with 5 mm dual and reverse probe heads at 400 MHz (^1H) and 100 MHz (^{13}C). The ^1H 1D spectra were recorded with inversion–partial recovery of water, using 64K points, sweep width (SW) = 3623 Hz, frequency (SF) = 400.137 MHz. The ^{13}C 1D spectra were acquired using 32K points, SW = 22 727 Hz, SF = 100.623 MHz. Data processing was by exponential multiplication with 2 Hz line broadening. HC COSY spectra were acquired as 512 × 1K complex points zero filled to give a data matrix consisting of 1K × 1K points, using the TPPI mode in the F_1 domain. Parameters for the F_1 domain were $SF_1 = 100.622$ MHz, $SW_1 = 150.573$ ppm and those for the F_2 domain were $SF_2 = 400.137$ MHz, $SW_2 = 2336$ Hz. Normal and long-range HC COSY spectral data were processed with square-sinusoidal multiplication prior to both Fourier transformations. NOESY spectra were recorded at 400.137 MHz using the TPPI mode in the F_1 domain, with a sweep width of 2325 Hz. Data were collected as 420 × 2K complex points, zero filled to 1K in F_1 to give a 1K × 1K contour plot and processed with sinusoidal multiplication prior to both Fourier transformations. Studies of the proton shift on dilution were carried out on a Jeol EX-270 instrument at 270 MHz (^1H); ^{13}C shifts were measured using a Varian Unity-500 instrument at 125 MHz. The spectra were referenced to dioxane as an external standard. All measurements were made at 303 K.

RESULTS

Spectral assignments

Assignments of the ^1H spectra for chlorpromazine hydrochloride were carried out in 99.93% D_2O at a concentration of 11.12 mmol kg^{-1} (below the literature[1] CMC of 19 mmol kg^{-1}). Chemical shift and coupling splitting pattern combined with nuclear Overhauser enhancement measurements allowed the unambiguous assignment of the protons. Irradiation of the α-protons of the side-chain allowed a distinction to be made between H-9 and H-6 by observation of the nuclear Overhauser effect. Values of the coupling constants (Table 1) were measured directly for the ring protons and calculated using the simulation program LAOCOON for the protons of the chain. The values obtained for the latter confirmed the preferred *gauche-trans* conformation of the alkyl chain,[12,13] which allows the hydrophobic region of the molecule to be protected more adequately from the aqueous environment. The chemical shifts at concentrations of 73.60 and 4.856

Table 2. ^{13}C and ^1H chemical shifts of chlorpromazine hydrochloride at concentrations above and below the CMC in ppm.

	$\delta^{13}C$		δ^1H	
Position	4.858 mmol kg^{-1}	73.60 mmol kg^{-1}	4.858 mmol kg^{-1}	73.60 mmol kg^{-1}
1	117.16	116.74	6.93	6.59
2	134.02	133.82		
3	123.75	123.33	6.94	6.51
4	129.03	128.74	7.06	6.56
4a	124.64	124.23		
5a	125.81	125.34		
6	128.46	128.36	7.19	6.84
7	124.47	124.36	7.00	6.77
8	128.88	128.87	7.24	7.08
9	117.42	117.40	6.97	6.73
9a	145.02	144.19		
10a	146.75	146.80		
α	44.35	44.33	3.91	3.54
β	22.09	22.19	2.06	1.81
γ	56.28	55.83	3.10	2.82
Me	43.40	43.36	2.66	2.48

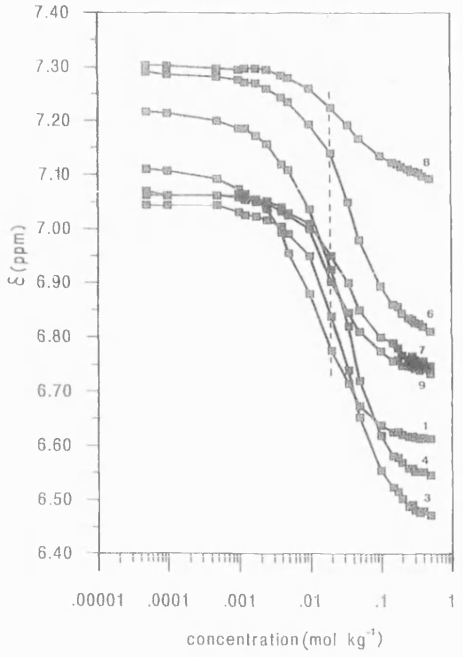

Figure 1. Chemical shift, δ, as a function of log (concentration) for aromatic protons of chlorpromazine hydrochloride in D_2O Dashed line, literature value of CMC.

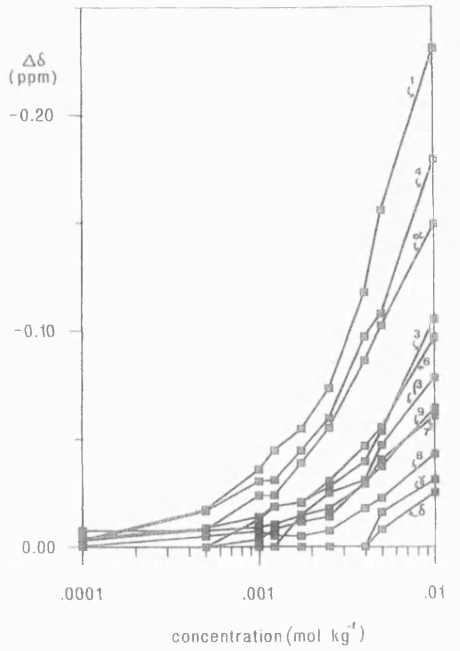

Figure 2. Change of chemical shift, $\Delta\delta$, as a function of log (concentration) for the aromatic and aliphatic protons of chlorpromazine hydrochloride in D_2O over the pre-CMC concentration range.

mmol kg^{-1} (above and below the CMC) are given in Table 2 and show a significant dependence of chemical shift on solution concentration.

Assignments of the ^{13}C spectra were made on a sample of concentration 73.60 mmol kg^{-1} using two-dimensional NMR with inverse detection. A two-dimensional CH COSY plot allowed the assignment of the proton-bearing carbons whilst a long-range CH COSY plot allowed the assignment of the quaternary carbon atoms. The chemical shifts given here for the C-4, C-6 and C-8 carbons differ markedly from those previously reported by Patra *et al.*[15] and Jovanovic and Biehl[16] from coupled ^{13}C spectra in D_2O and DMSO, respectively, at concentrations exceeding 1 mol dm^{-3}. However, these differences can be explained by the concentration dependence of the relative position of the carbon signals as seen from a comparison of the values given in Table 2 for solutions of concentrations 73.60 and 4.856 mmol kg^{-1}.

Concentration dependence of chemical shifts

The variation of the chemical shift, δ, of the aromatic protons with concentration as measured from spectra recorded over a very wide concentration range (5×10^{-5}–0.5 mol kg^{-1}) are presented in Fig. 1. Particular attention was given in investigations of the chemical shift in the region below the literature value of the CMC. Figure 2 shows the change of chemical shift ($\Delta\delta$) of all of the protons, including those of the side-chain, over the concentration range 10^{-4}–10^{-2} mol kg^{-1}, relative to the chemical shift in the most dilute solution (assumed to represent that of the free monomer). It is clear from these figures that the changes of shift of the protons of the ring bearing the Cl substituent (protons in positions 1, 3 and 4) are significantly larger than those of the unsubstituted ring and are appreciable at concentrations extending down to at least one tenth of the CMC. As all the external factors are maintained

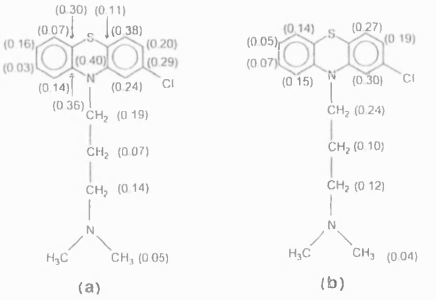

Figure 3. Schematic structures of chlorpromazine hydrochloride showing change in chemical shift over the pre-CMC concentration range. (a) Carbon shift over the range 0.9–18 mmol kg^{-1}; (b) proton shift over the range 0.05–18 mmol kg^{-1}.

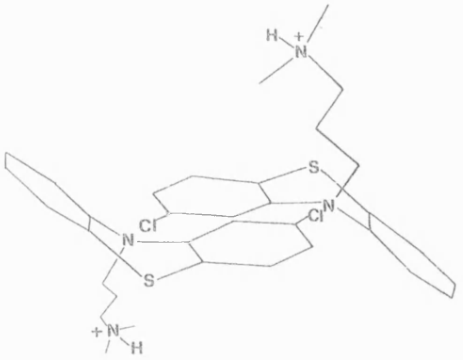

Figure 4. Schematic representation of the stacking of two molecules of chlorpromazine.

constant, it may be concluded that the chemical shifts must signify changes in the environment of the protons arising from the association of the chlorpromazine molecules. Shifts of similar magnitude were noted for the carbon atoms of this ring over a similar wide range of concentration. Figure 3 summarizes the shifts for each proton and carbon atom as measured from the difference between the constant value obtained in very dilute solutions and the values at the CMC.

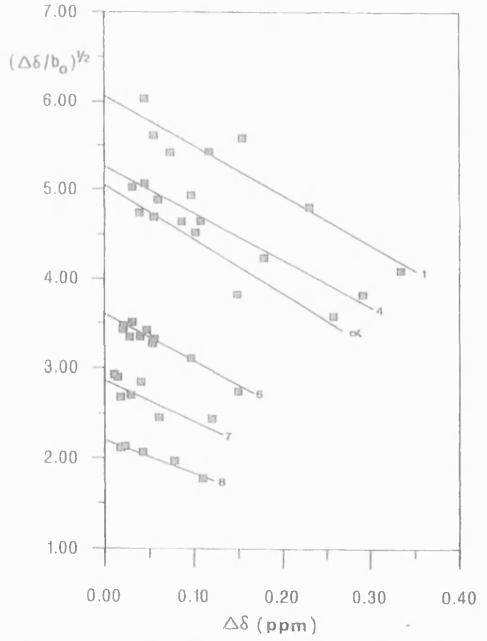

Figure 5. Chemical shift data for the protons of chlorpromazine hydrochloride below the CMC plotted according to Eqn (2).

DISCUSSION

Downfield shifts of ^1H resonances on dilution have been reported previously in studies of the association of nucleotides[17] and azo dyes[18,19] and attributed to parallel rather than coplanar assocation of the molecules. The phenothiazine ring system is V-shaped about an axis from the N to S atoms with an internal angle of approximately 155°. A concave to convex vertical stacking of the aggregate with the alkyl side-chains on alternate sides of the stack has been suggested previously by Florence and Parfitt,[9] and such an arrangement of molecules would appear to be both thermodynamically and sterically stable. Inspection of the relative magnitude of the changes in chemical shifts of aromatic protons and carbon atoms now provides more information on the possible spatial orientations of the stacked molecules. The greater involvement of the chlorinated rings in the association process suggests an offset orientation of the molecules of the stack with maximum overlap of these rings rather than the perfect alignment of the tricyclic ring system as originally envisaged (see Fig. 4).

Figure 2 shows that significant changes of the chemical shifts of the aromatic protons of chlorpromazine occur over a wide concentration range commencing at a concentration well below the literature value of the CMC. These results show clear evidence of premicellar association, supporting the findings from other experimental techniques.[4–8]

The proton chemical shifts over the concentration range up to the CMC were analysed using the method proposed by Dimicoli and Hélène[20] to quantify the extent of the association over this concentration region. For a stepwise association process in which multimers b_n are formed according to the equilibrium

$$b_1 + b_1 \rightleftharpoons b_2$$
$$b_2 + b_1 \rightleftharpoons b_3$$
$$\vdots$$
$$b_{n-1} + b_1 \rightleftharpoons b_n$$

it was shown that

$$\Delta\delta/b_1 = 2K\Delta\delta_{b_2} \quad (1)$$

$$(\Delta\delta/b_0)^{1/2} = (K/2\Delta\delta_{b_2})^{1/2}(2\Delta\delta_{b_2} - \Delta\delta) \quad (2)$$

In Eqns (1) and (2), $\Delta\delta = \delta - \delta_{b_1}$ where δ is the observed chemical shift and δ_{b_1} is that of the free monomer; $\Delta\delta_{b_2} = \delta_{b_2} - \delta_{b_1}$ where δ_{b_2} is the chemical shift of the dimer; b_0 and b_1 represent the total concentration and the free monomer concentration respectively; and K is the association constant, assumed to be identical for each association step. The assumptions underlying the application of the method of data treatment are that the magnetic anisotropic effects of neighbouring molecules are additive and that only the anisotropy of nearest neighbours need be considered. In the application of Eqns (1) and (2) it was assumed that the constant value of δ at low concentration was an adequate representation of the chemical shift of the monomer, δ_{b_1}.

Plots of $(\Delta\delta/b_0)^{1/2}$ against $\Delta\delta$ for the aromatic protons were linear (Fig. 5) in accordance with Eqn (2),

and a mean value of $K = 20.0 \pm 8.8$ kg mol^{-1} was derived from the gradient $(K/2\Delta\delta_{b2})^{1/2}$ and intercept on the abscissa $(2\Delta\delta_{b2})$. The percentage of drug existing as free monomer at the CMC was calculated to be 55% from Eqn (1) using the mean K value. Previous detailed examinations[5,6,8] of the premicellar association of phenothiazine drugs have concentrated on promethazine hydrochloride. A reasonable fit of calorimetric data for this drug was obtained using an association model similar to that used here in which the stepwise equilibrium constants were assumed to be of equal magnitude. The reported values[5] of K increased from 12 to 38 kg mol^{-1} with increase in electrolyte concentration from 0.1 to 0.6 mol dm^{-3} NaCl. The Cl substituent on the tricyclic ring system of chlorpromazine imparts a greater hydrophobicity compared with promethazine and consequently should result in higher values of K. The value obtained in this study for chlorpromazine in the absence of electrolyte is thus of the magnitude expected for this drug.

Acknowledgement

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REFERENCES

1. D. Attwood and A. T. Florence, *Surfactant Systems*, Chapt 4, Chapman and Hall, London (1983).
2. D. Attwood, D. Doughty, V. Mosquera and V. Perez Villar, *J. Colloid Interface Sci.* **141**, 316 (1991).
3. D. Attwood, R. Blundell and V. Mosquera, *J. Colloid Interface Sci.* **157**, 50 (1993).
4. D. Attwood, E. Boitard, J. P. Dubès and H. Tachoire, *J. Phys. Chem.* **96**, 11018 (1992).
5. D. Attwood, P. Fletcher, E. Boitard, J.-P. Dubès and H. Tachoire, *J. Phys. Chem.* **94**, 6034 (1990).
6. D. Attwood, E. Boitard, J.-P. Dubès and H. Tachoire, *Colloids Surf.* **48**, 35 (1990).
7. D. Attwood, N. A. Dickinson, V. Mosquera and V. Perez Villar, *J. Phys. Chem.* **91**, 4203 (1987).
8. W. Wan-Bahdi, H. Mwakibete, D. M. Bloor, R. Palepu and E. Wyn-Jones, *J. Phys. Chem.* **96**, 918 (1992).
9. A. T. Florence and R. T. Parfitt, *J. Phys. Chem.* **75**, 3554 (1971).
10. J. Barbe and A.-M. Chauvet-Monges, *C.R. Acad. Sci. Ser. C*, **279**, 935 (1974).
11. B. A. Soltz, J. Y. Corey and D. W. Lassen, *J. Phys. Chem.* **83**, 2162 (1979).
12. G. Fronza, E. Ragg and R. Mondelli, *Actual. Chim. Ther.* **8**, 245 (1981).
13. E. Ragg, G. Fronza and R. Mondelli, *J. Chem. Soc., Perkin Trans. 2* **12**, 1587 (1982).
14. D. B. Staiger, R. J. Warren, J. E. Zarembo, A. Post and E. L. Anderson, *J. Heterocycl. Chem.* **18**, 101 (1981).
15. A. Patra, A. K. Mitra and A. K. Mukhopadhyay, *J. Indian Chem. Soc.* **59**, 660 (1982).
16. M. V. Jovanovic and E. R. Biehl, *J. Heterocycl Chem.* **21**, 1589 (1984).
17. P. O. P. Ts'o, *J. Am. N.Y. Acad. Sci.* **153**, 785 (1969).
18. D. J. Blears and S. S. Danyluk, *J. Am. Chem. Soc.* **88**, 104 (1966); **89**, 21 (1967).
19. T. Asakura and M. Ishida, *J. Colloid Interface Sci.* **130**, 184 (1989).
20. J.-L. Dimicoli and C. Hélène, *J. Am. Chem. Soc.* **95**, 1036 (1973).

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Geometry-based simulation of the hydration of small molecules

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The behaviour of water structure-makers and breakers is of practical importance in protein folding in aqueous solution (G. Nemethy, *Angew. Chem., Intl. Ed. Engl.*, 1967, **6**, 195), but the mechanism for this process is not fully understood (K. A. T. Silverstein, A. D. J. Haymet and K. A. Dill, *J. Am. Chem. Soc.*, 1998, **120**, 3166). A computer simulation has been developed, of water structure around solutes, using only hydrogen bond geometry data rather than traditional forcefield methods. The simulation builds networks of hydrogen bonded water around structure-makers such as sulfate and phosphate, but not around structure-breakers such as urea and guanidinium. We have tested the software with a preliminary set of 17 varying solutes and our results are generally consistent with existing practical data.

Introduction

On the basis of volume and entropy measurements, Frank and Evans found that non-polar solutes affect the structure of the water around them, essentially making it more ice-like.[1] They also suggested that some molecules might act in the opposite way, making the water more mobile than bulk water. This 'iceberg' theory has often been misinterpreted, but has since become the standard model of hydrophobic hydration.[2] The concept was taken a stage further[3] when Frank and Wen classified various small ions as either structure-makers or structure-breakers. They also estimated that the individual water structures were very short-lived, with a half-life assumed to be 10^{-10}–10^{-11} s, and referred to these as 'flickering clusters' to distinguish them from solid 'icebergs'.

The term hydrophobicity was introduced by Kauzmann,[4] as an interpretation of protein denaturation using Frank's theories. He suggested that the attractive interactions between non-polar molecules in aqueous solution are promoted by the solvent as a result of destructive overlap of hydration shells releasing structured water into the bulk. Hydrophobic effects play a pivotal role in protein folding,[5] helical stabilisation,[6] micelle formation,[7] and the action of surfactants.[8] Strong structure-breaking molecules such as urea and guanidinium will cause protein denaturation,[9] in which the protein unfolds and may precipitate out of solution. This process is governed by the effect these structure-breakers have on the water molecules around them.[10] In contrast, structure makers such as sulfate ions can be used to assist the folding or renaturation of proteins.[11]

Water molecules are potentially tetrahedral, with two hydrogen bond donor and two acceptor positions. Information about the geometric preferences of aggregates of these tetrahedra may be found from clathrate hydrates, solid crystalline complexes in which a small non-polar molecule is encaged in a regular network of five- and six-membered rings of hydrogen-bonded water molecules.[12] Rings of water molecules have also been proved to exist near the surface of proteins,[13] at DNA interfaces,[14] and in the hydration shells of DNA–drug complexes,[15] suggesting that water in these environments has much in common with the arrangement in clathrate hydrates. The three most common cavity types found in clathrates have also been found in the gaseous phase[16] around small ions such as Cs^+. The existence of such structures in the liquid state has been the subject of speculation for many years but recent work[17] involving the hydrophobic hydration of krypton using extended X-ray absorption fine-structure (EXAFS) spectroscopy has shown that the liquid to solid phase transition produces a clathrate hydrate, indicating that Frank's 'structuring' of liquid water by non-polar solutes may be very similar to clathrate hydrate structures.

Quantum mechanical calculations on randomly generated arrangements of water molecules have shown that rings are more stable than chains,[18] and it has been experimentally verified that the predicted minimum energy structures are found in bulk water.[19] We have developed software to model possible hydrogen bonded water structure around different solute molecules, using a purely structural model of water, with ring formation as a primary feature. As we are only using hydrogen bond geometries to constrain the program, it is important that the values used are realistic.

Method

We have surveyed the hydrogen-bond geometries of 14 ice and 11 clathrate crystal structures and 60 hydration structures of small biological molecules, the latter from the Cambridge Crystallographic Data Centre,[20] using neutron scattering data where available as these include the deuterium positions. Information was taken from diffraction studies of 18 carbohydrates and hydration structures of nucleosides, nucleotides, purines, pyrimidines and other molecules where hydrogen bonds with water have been observed. More restricted searches have been published for carbohydrates[21] and small-molecule components of the nucleic acids.[22]

Our survey has shown a narrow distribution of O–H···O bond lengths in these different environments [Fig. 1(a)], in the range 2.8 ± 0.1 Å, the value used in the simulation. The

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hydrogen bond angle distribution is wider [Fig. 1(b)] and the angle constraint our program uses for the ring-forming hydrogen bonding step is normally set in the range 180 ± 25° to reflect the experimental data. Measurements of pair correlation functions in liquid water indicate that our chosen values are realistic,[24] with the O–O distance very close to 2.8 Å and the O–H distance at 1.8 Å. This implies that the O–H–O bond angle is near to 180°, since the O–H covalent bond length is close to 0.96 Å.

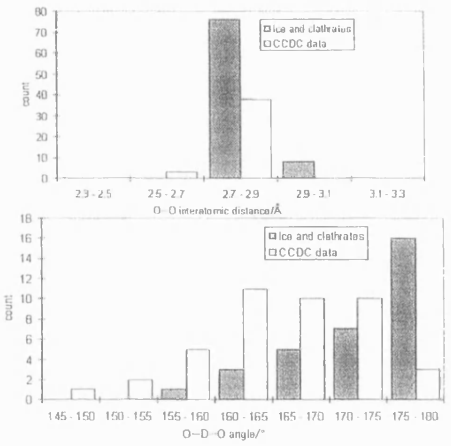


Fig. 1 Survey of hydrogen bond geometries in ices and clathrate hydrates, and small biological molecules from the CCDC. (a) Distribution of O···O interatomic distances for ices and clathrate hydrates (grey bars) and hydrates of small biological molecules from the CCDC (clear bars). (b) Distribution of O–D···O bond angle for ices and clathrate hydrates (grey bars) and hydrates of small biological molecules from the CCDC (clear bars).

Solutes without internal rotation

In our model, a random hydrogen-bonding site (X) on a solute molecule is chosen. A water molecule is added at this site, such that the X–H–O angle is 180° and the X–O distance is 2.8 Å. A test is carried out, to determine whether the water oxygen is the correct distance for H-bonding to any other site (Y) in the molecule; if this test is passed, the water is rotated in 10° steps, to determine whether a hydrogen bond can be formed, satisfying the O–H–Y angle criterion of 180 ± 25°. If this test is also passed, a ring is formed and another site is chosen at random, for the whole sequence to be repeated. If either the proximity or angle test fails, no ring is formed and another water is bonded to the first water. The proximity test is again carried out, between the terminal water oxygen and any other potential H-bonding site. If this test is passed, the terminal water is rotated as before, testing for possible H-bonding and ring formation. If either test fails, the penultimate X–H–O bond is rotated in steps, testing after each rotation for proximity of the terminal oxygen to a potential H-bond site and if appropriate for angle, as before. If no ring is formed, a further water is added and the whole process is repeated, if necessary making a complete stepwise rotation around the penultimate O–H–O bond, then a one-step rotation around the X–H–O bond, followed by a complete rotation around the penultimate O–H–O bond and so on until the X–H–O bond has rotated through 360°. In this way the whole of the space is tested for potential ring-formation. In a long chain of waters every X–H–O or O–H–O bond is rotated, except that the most recently added water is rotated only when the proximity test is passed. Once a ring is formed, the ring is retained without further adjustment and the process is restarted.

The speed at which the simulation occurs is directly affected by the chosen rotational increment. If each increment is 10°, there are 36 steps to achieve complete rotation and the time taken is proportional to 36^n where n is the number of bonds around which rotation occurs. Despite the very large number of calculations required, on a 400 MHz PC the simulation takes only a matter of seconds when rotation occurs around four bonds or less, but rotation around five bonds takes over a minute and rotation around six bonds takes about one hour (see below). In practice, there is no need to add more than four waters, since further water molecules will tend to form rings with the first added water in the chain, excluding the solute, as shown in Fig. 2 below.

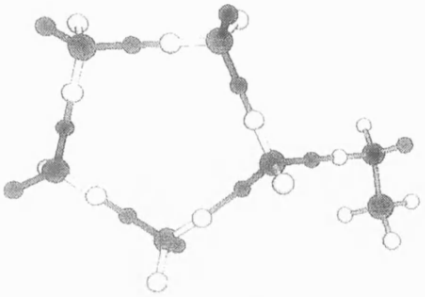
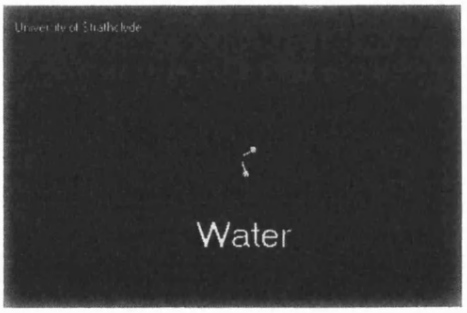


Fig. 2 Methylamine with a ring of water molecules attached by just one hydrogen bond.

The structures of all the solute molecules used in the simulations were taken from diffraction studies and the lone-pair electrons were placed and oriented by examining the structures of the solute molecules and the positions of water molecules from published hydration studies of the solute, where available.

The solutes are described in terms of three-dimensional coordinates and the locations, either hydrogens or lone pairs, which allow hydrogen bonding. For each hydrogen bonding opportunity, a vector is stored specifying the direction of the hydrogen bond. The coordinates, together with the van der Waals radii of the atoms, specify the size



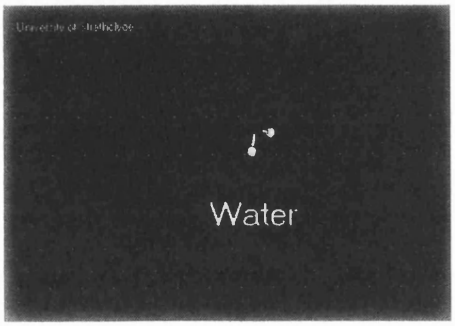
AVI motion picture (2 Mb)
GIF motion picture (0.1 Mb)

Video 1

and shape of the molecule and the regions into which the water cannot encroach.

Results

With a water molecule as the initial 'solute', the simulation rapidly produces a cluster of mainly five-membered rings (Video 1). Using a 400 MHz PC, this simulation runs in 90 s for a total of 50 water molecules. Each simulation is different, although it is not possible to show this in the video. In Video 2 the simulation of the formation of the first water ring is slowed down to show how the rotations occur. Table 1 describes the colour notation used.



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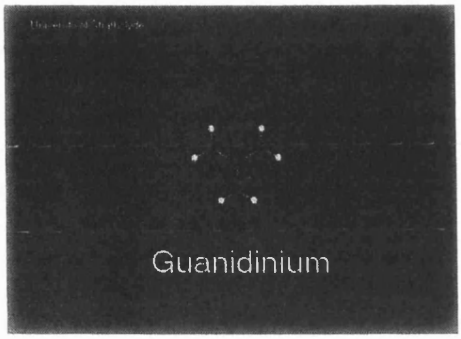
Video 2

If the water chain reaches a length of four molecules and the rotations do not detect any hydrogen bonding opportunities, as for the structure-breakers urea and guanidinium, then that particular water chain is 'dissolved' and the process restarted, reflecting a 'flickering cluster' with a very short life. (Video 3) In this way a network of hydrogen-bonded water molecules is only constructed around the solute molecule if rings can be formed. With the structure-breaking solutes, many thousands of water molecules can be added and then 'dissolved' without a longer-lasting structure being formed.

In contrast, rings of water readily form around the structure-making solutes sulfate (Video 4) and

Table 1 Colour scheme for all videos and still images from the simulation program

Structural element	Colour
Carbon	Blue
Nitrogen	Darker blue
Oxygen	Red
Sulfur	Yellow
Hydrogen	White
Lone pair 'pseudoatoms'	Dark blue
Covalent bond	Purple
Hydrogen bond	Green



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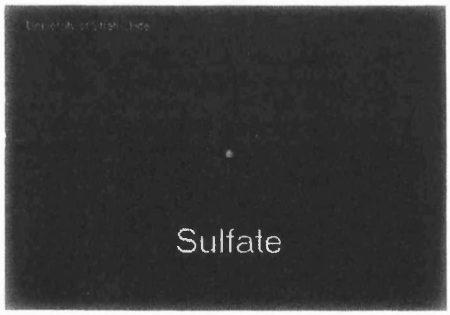
GIF motion picture (0.4 Mb)

Video 3

phosphate, leading to large networks around these species. The recruitment of the first water molecule is random among all the available H-bonding sites on the solute. Subsequent water molecules may be added at any of the three H-bonding sites on the end-terminal water as the chain is formed, so there is a substantial random element in the formation of the structured hydration layer. Repetition of the simulation gives different results every time, but the structure-makers always produce rings of water molecules, which are allowed to persist. For both sulfate and phosphate we assume three H-bonding opportunities for each oxygen, disposed at three corners of a tetrahedron. There is a question concerning the orientation of these H-bonds, relative to the remaining three oxygens. We have assumed that the molecule will adopt a non-eclipsed conformation (Fig. 3) and this is supported by X-ray data on hydrated sulfates.[24]



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Video 4

Having established that the simulation gives sensible results, in accord with the measured behaviour of the solutes, we extended the simulation to a range of molecules which we anticipated would have interesting interactions with water. The simple alkylamines have been

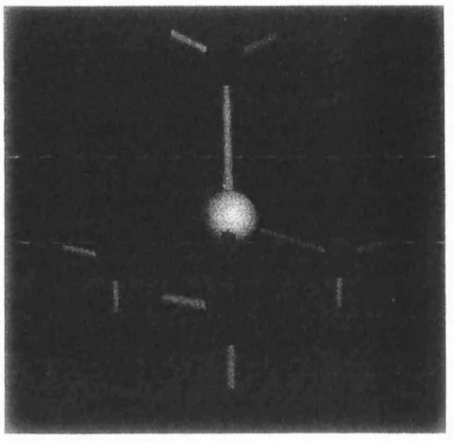
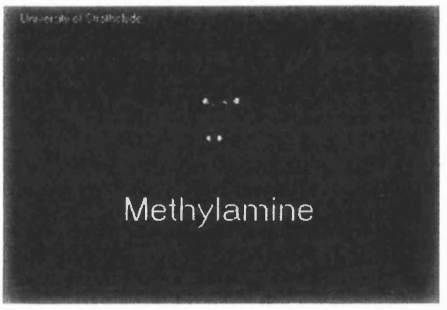


Fig. 3 Structure of the sulfate ion. Lone pairs are shown in blue.

shown to produce clathrates in which the primary amino group is H-bonded into the clathrate cage.[25] To model this efficiently using our software required one small change, to avoid the added water molecules orientating H-bond vectors towards the alkyl group, since this does not happen in reality. In practice only the first water molecule has to be controlled in this way, subsequent additions being directed by the first one. The result is a strong tendency to form cages around the alkyl group, similar to the clathrate structure (Video 5).



AVI motion picture (2 Mb)
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Video 5

Phenol and catechol are both structure-breakers.[26] In both cases, the hardest part of the simulation lies in deciding how the structure should be represented, in terms of the H-bond vectors. We assumed, in the case of phenol, that one lone pair is conjugated with the benzene ring and that the other lone pair and the hydroxy proton are in the plane of the ring. Water molecules added to this system cannot form a ring with four water molecules [Fig. 4(a)] and further addition of water molecules results in water-only rings forming. In the case of catechol, we assumed that one OH hydrogen is H-bonded to the adjacent oxygen and that both oxygens have one lone pair conjugated with the ring. This results, again, in one H-bond donor and one acceptor and again added waters cannot form a ring [Fig. 4(b)].

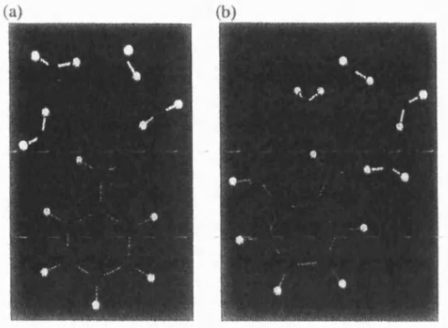
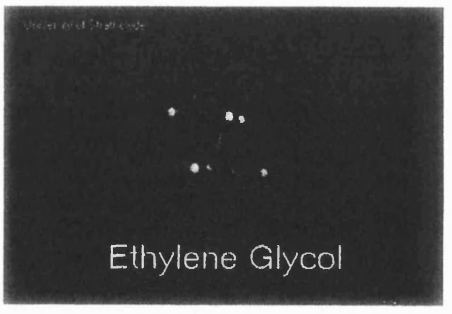


Fig. 4 Four water molecules attached to phenol (a) and catechol (b) showing that a ring cannot be formed.

Solutes with internal rotation

Given that there are practical limits on the number of bonds about which rotation can be allowed to occur, very large or complex solutes are beyond the presently available computing power (a 400 MHz PC). However, solutes can be modelled in which up to four internal bonds are allowed to rotate, with up to two added waters, in a tolerable time span. Ethanediol (1,2-dihydroxyethane, ethylene glycol) is a known structure-breaker,[27] with rotation possible about three internal bonds. We are therefore able to add up to three water molecules in reasonable computer time. When we do this, using rotational increments of 10°, a ring is sometimes formed with two water molecules but most often with three: we assume that the model misses with two waters when chance puts the potential bond between two 10° steps. Presumably, if we were able to use sufficiently small rotational increments, a ring would be formed with two waters every time. However, a reduction to even 5° increments would increase the computation time for rotation around five bonds by a factor of $72^5/36^5$ *i.e.* it would take 32 times as long (2^5). With the existing processor this is not feasible. Reducing the angle increment from 10 to 1° would take 10^5 times as long for rotation around five bonds.

Video 6 shows the start of the search with ethanediol and one water molecule: the process takes too long to show the addition of the second and third waters. Video 7 shows ring



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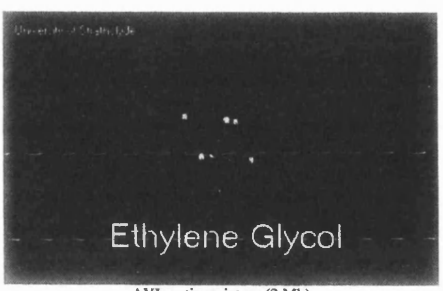
Video 6



AVI motion picture (2 Mb)
GIF motion picture (0.1 Mb)

Video 7

formation with three waters and the subsequent build up of more rings to give a complex 3D structure.

With either two or three waters, the ring formed with ethanediol has the solute in a *gauche* conformation (Fig. 5). Since the simulation takes no account of conformational energy, the preferred conformation is fortuitous, but may indicate a driving force for the adoption of the *gauche* conformation, rather than the *trans*, in aqueous solution. Since ethanediol is a structure-breaker (and is used as 'antifreeze'), the formation of rings implies that the geometry of the water structure so formed is not compatible with the geometry of structures formed in the absence of solute.

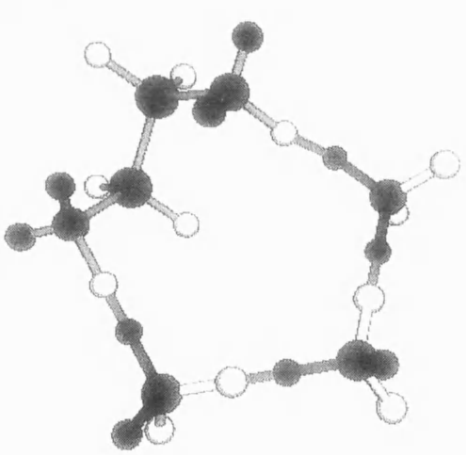


Fig. 5 Ethanediol with three added water molecules.

Ethanolamine behaves in a similar manner. The hydroxy group again adopts a *gauche* position relative to the amine. In both these cases, the internal torsion angle in the solute (O–C–C–O or O–C–C–N) when a ring is formed is always close to 60°, *i.e.* a non-eclipsed conformation (Fig. 6).

We also ran the simulation using 1,3-propanediol as solute. In this case rings formed with either one or two added water molecules. The diol again tends to be oriented close to the gauche conformation. The two internal torsion angles (O–C–C–C and C–C–C–O) are close to 60° in the structures with one and two conjugated waters (Fig. 7).

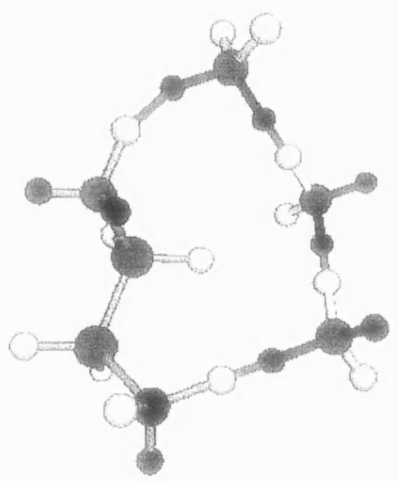


Fig. 6 Ethanolamine forming a ring with three added water molecules.

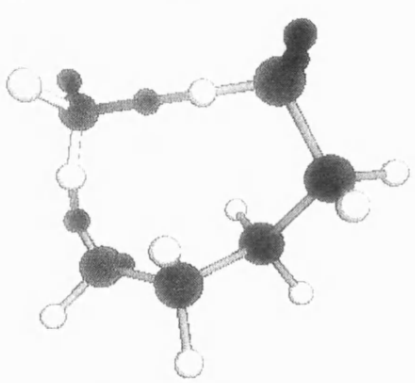


Fig. 7 1,3-Propanediol showing a cyclic structure involving just one water molecule.

Discussion

The simulations we have run have given results which largely conform with the known behaviour of the solutes, as measured by, for example, the depression in the freezing point of water. The widely used structure-breakers urea and guanidinium produce non-cyclic, short-lived structures which conform to the notion of 'flickering clusters', while the important structure makers sulfate and phosphate produce complex three-dimensional water structures, similar to ice. Phenol and catechol behave similarly to the other known structure-breakers, while methylamine readily produces cages around the alkyl group, in accord with the clathrate structure.

The flexible diols are more difficult to assess in the initial stage of hydration, as shown here. Further addition of water molecules is required, to build up more complex structures. These structures will require the development of new methods of assessment, to compare their geometry with that of the ices and clathrates, in a quantitative manner. We are

working on methods to accomplish this, since it could be argued that the very simple structures depicted here could be modelled by physical means. The strength of the computer method lies in the ability to add large numbers of waters and to assess the nature of the structures so formed.

It is central to our approach that rings are more stable than chains, as predicted by theory, and that the higher levels of 3D structure are more stable than simple rings: the analogy with the chemistry of sp^3 carbon is that the most stable structure is diamond, which is geometrically analogous to many of the ices and clathrates, except that diamond does not have the cavities. We would assume that the random transfer of thermal energy would relatively easily break a chain, with little probability of re-formation, whereas a ring requires two breakages and higher order structures are even less likely to be broken up. The overall effect, we assume, is not that the ice-like structures persist indefinitely, but that they have a longer lifetime than lower-order structures. The increased statistical probability of higher order is sufficient to change the properties of the solution. The results shown here are the first stage in the simulation of larger structures, involving large numbers of water molecules, which may show differences in hydration between different solutes which are not amenable to easy inspection.

The applicability of our approach is potentially wide-ranging. It may be possible, for example, to predict the formation of clathrates in situations of practical utility, such as in the development of new aerosol formulations where a hydrophobic gas may come into contact with water and block the delivery device. Of broader significance, however, is the potential to model the interactions of biochemically and pharmacologically important molecules with water. Adrenaline and noradrenaline, for example, both have structure-breaking geometry in the catechol moiety and an ethanolamine unit at the other end of the molecule. Whether this is significant in the context of their interactions with adrenoceptors remains to be seen; it is possible that a local, specific 'denaturing' effect on the receptor protein is central to their biological action. With increased computing power and refinement of the present software, it will be possible to simulate the hydration layer of these more complex molecules.

Electronic Supplementary Information

Programming details are available in a separate document. Click here to access them.

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References

1 H. S. Frank and M. W. Evans, *J. Chem. Phys.*, 1945, **13**, 507.
2 W. Blokzijl and J. B. F. N. Engberts, *Angew. Chem., Int. Ed. Engl.*, 1993, **32**, 1545.
3 H. S. Frank and W. Y. Wen, *Discuss. Faraday Soc.*, 1957, **24**, 133.
4 W. Kauzmann, *Adv. Protein Chem.*, 1959, **14**, 1.
5 K. A. Dill, *Biochemistry*, 1990, **29**, 7133.
6 M. A. Jiminez, C. Carreno, D. Andreu, F. J. Blanco, J. Herranz, M. Rico and J. L. Nieto, *Biopolymers*, 1994, **34**, 647.
7 C. Tanford, *The hydrophobic effect, formation of micelles and biological membranes*, 2nd edn., Wiley, New York, 1980.
8 A. T. Florence and D. Attwood, in *Physicochemical principles of pharmacy*, 2nd edn., Macmillan, London, 1988, p. 173.
9 D. B. Wetlaufer, S. K. Malik, L. Stoller and R. L. Coffin, *J. Am. Chem. Soc.*, 1964, **86**, 508.
10 C. Tanford, *J. Am. Chem. Soc.*, 1964, **86**, 2050.
11 K. D. Collins, *Biophys. J.*, 1997, **72**, 65.
12 G. A. Jeffrey and W. Saenger, *Hydrogen bonding in biological systems*, Springer-Verlag, Berlin, 1991.
13 M. Gerstein and R. M. Lynden-Bell, *J. Phys. Chem.*, 1993, **97**, 2982.
14 L. A. Lipscomb, M. E. Peek, F. X. Zhou, J. A. Bertrand, D. VanDerveer and L. D. Williams, *Biochemistry*, 1994, **33**, 3649.
15 B. Schneider, S. L. Ginell and H. M. Berman, *Biophys. J.*, 1992, **63**, 1572.
16 A. W. Castleman, *J. Phys. Chem.*, 1991, **95**, 8442.
17 D. T. Bowron, A. Filipponi, M. A. Roberts and J. L. Finney, *Phys. Rev. Lett.*, 1998, **81**(19), 4164.
18 J. K. Gregory and D. C. Clary, *J. Phys. Chem.*, 1996, **100**, 18014.
19 K. Liu, M. G. Brown, C. Carter, R. J. Saykally, J. K. Gregory and D. C. Clary, *Nature*, 1996, **381**, 501.
20 F. H. Allen and O. Kennard, *Perspect. Comput.*, 1983, **3**, 28.
21 T. Steiner and W. Saenger, *J. Am. Chem. Soc.*, 1992, **114**, 10146.
22 B. Lesyng, G. A. Jeffrey and H. Maluszynska, *Acta Crystallogr., Sect B*, 1988, **B44**, 193.
23 A.K. Soper, F. Bruni and M.A. Ricci, *J. Chem. Phys.*, 1997, **106**, 247
24 G. M. Brown and R. Chidambaram, *Acta Crystallogr., Sect B*, 1969, **B25**, 676.
25 R. K. McMullan, T. H. Jordan and G. A. Jeffrey, *J. Chem. Phys.*, 1967, **47**, 1218.
26 M. Nakahara and Y. Yoshimoto, *J. Phys. Chem.*, 1995, **99**, 10698.
27 F. Corradini, L. Marcheselli, L. Tassi and G. Tosi, *J. Chem. Soc., Faraday Trans.*, 1993, **89**, 123.

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Review Article

Water structure theory and some implications for drug design

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Abstract

The development of theories of water structure has been hindered in the past by the difficulty of experimental measurement. Both measurement and computer modelling studies have now reached the stage where theoretical treatments of water structure are converging to a broadly acceptable model. In current understanding, water is a mixture of randomly hydrogen-bonded molecules and larger structures comprised of tetrahedral oxygen centres which, when hydrogen-bonded to each other, lead to five-membered and other rings which can aggregate to form three-dimensional structures. Evidence is taken from studies of the ices, from clathrates and other solid solutions, as well as from liquid solutions, that certain motifs occur very frequently and have relatively high stability, such as the $(H_2O)_{20}$ cavity-forming structure known from studies on clathrates. The implications of recent models of water structure for an understanding of biological events, including the interactions of drugs with receptors, are profound. It is becoming clear that modelling of aqueous solutions of any molecule must consider the explicit interactions with water molecules, which should not be regarded as a continuum: water itself is not a continuum. Solute molecules which possess hydrogen-bonding groups will provoke the formation of further hydrogen-bonding chains of water molecules: if these can form rings, such rings will tend to persist longer than chains, giving the solute a secondary identity of associated water which may play a role in molecular recognition. Solutes that do not have hydrogen-bonding capability, or regions of solutes which are non-polar, may also produce partial cage-like water structures that are characteristic of the solute.

The classification of many solutes as structure makers or structure breakers has relevance to the interactions between ligands and large biomolecules such as proteins. While it is generally accepted that sulfate and urea, respectively structure maker and breaker, may alter protein conformation through effects on water, it has not been recognised that bioactive ligands, which also change the conformation of proteins, may do so by a related, but more selective, mechanism. Very early studies of cell contents suggested that the associated water might be different from bulk water, a concept that lost support in the mid-20th century. Current theories of water structure may invite a reappraisal of this position, given the observation that structuring may extend for many molecular diameters from an ordered surface.

Introduction

Water is the dispersion medium for all the biochemical reactions that constitute the living process and takes part in many of these reactions. Despite the chemical simplicity of the water molecule, its bulk properties are very peculiar and have attracted a large amount of scientific attention. Recent physico-chemical studies, allied to increasingly sophisticated computer simulations, have reached the stage where many of the old controversies have been resolved. Structuring in liquid water is being described with increasing confidence, in the pure liquid, at interfaces and in solutions. This review attempts to draw together much current understanding of water and to place the interactions of small molecules (e.g. drugs) and large molecules (e.g. receptors) into that context.

Early theories of water structure

The immiscibility of water with extruded cytoplasm from plant and animal cells led some mid-19th-century biologists to believe that water in the cytoplasm is bound

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to the macromolecular constituents (Nageli 1855; Kuhne 1864). The suggestion was made that water may contain solid particles, and in 1891 Vernon postulated that maximum density effects could be explained by the aggregation of water molecules (Davidson 1973). Some water in protein solutions and in living cells refused to freeze at temperatures as low as −20°C (Rubner 1922).

In the 1930s Gortner and co-workers carried out experiments dissolving sucrose into water in various environments and determining how much had dissolved by measuring lowering of the freezing point (Gortner & Gortner 1934). They found that bound water, for example cell water, dissolved less sucrose than free water did and hence inferred that water in these situations was more structured than the bulk.

At the same time, the first X-ray (Morgan & Warren 1938) and infrared studies (Magat 1936) on liquid water were carried out, and this new perspective allowed Bernal & Fowler (1933) to advance the first plausible model for liquid water – the uniform continuum model. In this model, all oxygen atoms retain their four-coordination, but the hydrogen bonds are bent to such an extent that an instantaneous view from the central oxygen would see no order beyond the nearest neighbours. A random network model with soft hydrogen bonds implies a water network with apex-linked polygons in rings of 4-, 5-, 6- or more-membered rings, similar to ices and clathrates, but randomly arranged.

Even at this early stage the bound water theories met with considerable opposition. In a 1940 review, Blanchard compiled evidence against the bound water viewpoint on the basis of vapour-pressure measurements and the fact that ethylene glycol will distribute itself evenly between erythrocytes and external solution (Blanchard 1940). He also stated that water can be supercooled to −20°C and suggested that the complexity of the protein system made accurate determination of freezing point almost impossible. This review marked the end of the first phase of research into structured water as the membrane theory, where the cell was assumed to maintain a different composition from that of its surroundings by the use of sodium pumps located in the cell membrane, became generally accepted and the hydration theory of cell water was virtually abandoned.

Following on from work on the origin of negative partial entropies exhibited by various simple solutes in aqueous solution (Eley 1939), Frank & Evans (1945) introduced the concept of icebergs induced in water by solute molecules. On the basis of volume and entropy measurements, while studying the effects of non-polar molecules dissolving in water (noble gases, CO, methane, etc.), they found that these molecules affect the water around them making it less dense, at lower entropy and less mobile than bulk water, essentially making it more crystalline and more structured. They postulated that some species would act in the opposite way, making the water less structured and more dense than bulk. This was the first mixture model of liquid water. Subsequent models have generally been based either on this work or on Bernal & Fowler's continuum model (Bernal & Fowler 1933).

At the same time, Samoilov (1946) was investigating the nature of the interactions between water and ions in solution. Hall (1948), on the basis of acoustic relaxation in water, advanced the first detailed mixture model, based on the concept of water as a mixture of two distinguishable species. Pople (1951), working with the model proposed by Bernal & Fowler (1933) and his own studies into the properties of the hydrogen bond, suggested that hydrogen-bond bending could explain properties such as the temperature dependence of the dielectric constant of water.

The next major step forward for the structured water viewpoint occurred when Frank & Wen (1957) postulated that the existence of long-lived structures in liquid water was unlikely and that a more useful description might involve flickering clusters of hydrogen-bonded water molecules. They classified ions as either being structure makers or structure breakers, and suggested a mechanism by which this may occur. Basically, the ion is surrounded by three concentric regions: the innermost, in which all water is immobilised; the second in which water is less ice-like (i.e., more random than normal); and the third, normal water polarised in the normal way by the ionic field, which has become relatively weak this distance from the ion.

The ions originally classified by Frank & Wen were as follows:

structure makers: Li^+, F^-, Mg^{2+}, OH^-, $(n\text{-}C_4H_9)_4N^+$

structure breakers: K^+, Rb^+, Cs^+, Cl^-, Br^-, I^-, NO_3^-, ClO_4^-, SO_4^-

They postulated that the structure-breaking region was caused by the balance between the two competing orienting influences acting on any given water molecule in this region (i.e. the normal water influence from the outer zone and the spherically symmetrical ionic field dominating in the inner zone). They also attributed stronger structure-making ions with a high enough degree of incipient hydrolysis of the first water layer around the ion to produce hydrogen charge centres which would act as edge nuclei for cluster formation and stabilisation for clusters once formed. This increases the ice-likeness of the water in these environments.

Shortly afterwards, Kauzmann (1959) discussed the possible role of water in protein conformation and denaturation incorporating Frank and Evans' model. He reviewed the role of apolar group interactions or hydrophobic bonding in maintaining the tertiary structures of proteins, and suggested that comparative studies of aqueous and non aqueous solutions of simple analogues of apolar amino-acid side chains (alkanes, for example) may provide an insight into the peculiarities of water as a solvent. This sparked off a whole range of publications describing various aspects of hydrophobic bonding in biological systems.

Pauling (1959) proposed a model for the structure of water in which clathrate cages are present and may be occupied by interstitial non-bonded water molecules. This model was considered to be too crystalline (Stillinger 1980), and is not consistent with X-ray diffraction data, but less rigidly defined cavities, more disordered than the clathrates, would fit the experimental evidence available both in 1959 and today.

Most of the material arising from this vast amount of research in the 1960s and '70s is summarised in Felix Franks' 7-volume collection, *Water: a comprehensive treatise* (Franks 1972–1982).

At present, all models developed for the structure of liquid water still fall into two main classes: continuum models, originally proposed by Bernal & Fowler, and mixture models based on Franks' work. Speculation regarding the nature of molecular motions in water led to comparisons of various bulk and microscopic transport processes (e.g. viscosity, self-diffusion and dielectric and NMR spin-lattice relaxation). High-precision Raman studies of the hydrogen bonding modes in liquid water followed (Walrafen 1964), and infrared and Raman techniques have been applied to the study of intramolecular OH and OD stretching modes in H_2O and D_2O (Walrafen 1968; Senior & Verall 1969). This information was carefully analysed to deduce whether evidence suggested that water should be treated as a mixture and, although this was not fully resolved, mixture models were favoured. Frank & Evans' iceberg theory has now become accepted as the standard model of hydrophobic hydration (Blokzijl & Engberts 1993).

Structure-making and structure-breaking molecules

It has long been known that the native conformations of proteins, as assessed by enzyme activity, denaturation temperature and solubility, are stabilised by some solutes and destabilised by others (e.g. Collins & Washabaugh 1985). These effects are additive; a strongly denaturing (structure breaking) solute such as urea can be balanced by the action of a strongly stabilising (structure making) solute such as trimethylamine oxide. Two structure-breaking solutes will destabilise protein structure more than if either was used separately.

The structure-making (kosmotropic) or structure-breaking (chaotropic) action of solutes is determined in aqueous solution by:

A change in viscosity (structure breakers lower it)
The rate of exchange of water molecules (structure breakers lower energy of activation)
The longitudinal relaxation rate of water molecules as measured by NMR (structure breakers increase the rate).

The entropy of hydration is related to the structure-making and -breaking properties of solutes. Marcus (1986) estimated the entropy contribution of structured water effects by subtracting contributions due to compression, immobilisation and electrostatic effects from the standard molar entropies of hydration for a set of 50 ions. This structural entropy contribution is positive for structure breakers and negative for structure makers, and was found to be in accord with experimental findings despite the assumptions involved in the calculations.

The Hofmeister series, discovered over a hundred years ago (Hofmeister 1888) originates from the ranking of various ions based on their ability to precipitate hen egg-white proteins. This series has also been shown to affect the structuring (at one end of the series) or denaturing (at the other end of the series) of biological macromolecules in the same order (Collins 1997). The series shows opposite correlations for anions and cations with their degree of hydration.

$$\underset{\text{Strongly hydrated}\\\text{Protein stabilising}}{\text{citrate}^{3-} > \text{sulfate}^{2-} > \text{phosphate}^{3-} > F^- > Cl^- > Br^- > I^- > NO_3^- > ClO_4^-}\quad \underset{\text{Weakly hydrated}\\\text{Protein denaturing}}{}$$

$$\underset{\text{Weakly hydrated}\\\text{Protein stabilising}}{N(CH_3)_4^+ > NH_4^+ > Cs^+ > Rb^+ > K^+ > Na^+ > H^+ > Ca^{2+} > Mg^{2+} > Al^{3+}}\quad \underset{\text{Strongly hydrated}\\\text{Protein denaturing}}{}$$

Structure-breaking ions destroy the hydrogen-bonded water network in a manner which is similar to the effect of increased temperature or pressure (Leberman & Soper 1995). Anions hydrate more strongly than cations of the same ionic radius, as water hydrogens can approach about 0.8 Å more closely than the water oxygen. Small ions are strongly hydrated with small or negative hydration entropies creating local order. Large singly charged ions have larger positive entropies of hydration, and so act like hydrophobic molecules, their binding being dependent on van der Waals forces as well as their charge.

Very similar molecules often have totally opposite effects. Both *meta*- and *para*-hydroxybenzoic acids are structure makers, whereas the *ortho*-form is a structure breaker (Chatterjee & Seal 1992). Sulfate is almost exactly the same size and shape as perchlorate, but sulfate is a strong structure maker and perchlorate a strong structure breaker (Collins & Washabaugh 1985; Yokoyama et al 1992). The reasons for this still remain unclear.

The Walden product, which is the product of viscosity and conductivity at infinite dilution of a solution, has been shown to be a measure of the water-structuring activity of the solute. This has been used to quantify structure-making and -breaking effects of amino acids, in conjunction with viscosity and spectroscopic studies. Most amino acids exhibited some structure-breaking activity. L-Lysine, L-glutamic acid, L-aspartic acid and their salts showed stronger structure-breaking activity. Dextrose, however, behaved as a classic structure maker and reversed the structure-breaking action of L-lysine (Lutz et al 1994).

Pure water

Structure of the isolated water molecule

The structure of the isolated water molecule is known accurately from spectroscopic studies. The H—O—H angle and O—H bond lengths were first determined accurately by Darling & Dennison (1940). They found the O—H bond length to be 0.95718 Å and the H—O—H angle to be 104.523°. These values have since been validated by experimental (neutron scattering: Soper & Phillips 1986) and theoretical calculations (e.g. ab-initio: Goddard & Hunt 1974).

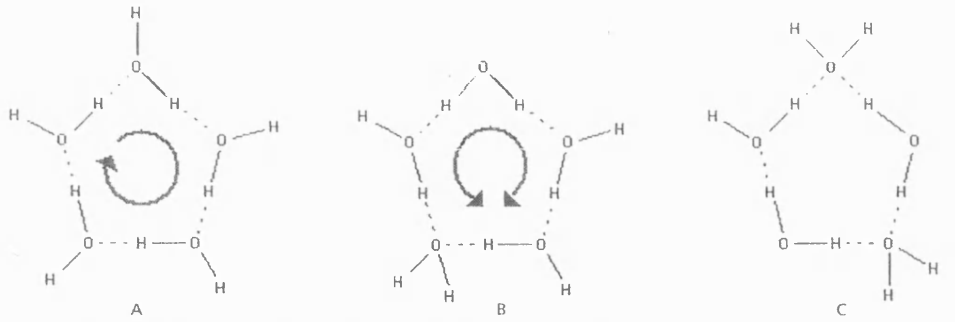

Figure 1 Three possible arrangements of cyclic hydrogen bonds: homodromic (A); antidromic (B); and heterodromic (C).

Each water molecule can form up to four hydrogen bonds in a tetrahedral arrangement: two acceptor bonds through the lone pairs and two donors through the hydrogens. This is a unique feature of the water molecule and leads to three-dimensional networks as, for example, in ice structures.

Cooperative effects

An important feature of hydrogen bonds is that one will tend to reinforce another. This is known as cooperativity, and allows large tetrahedral networks of hydrogen-bonded water to form. Cooperativity, or non-additivity of hydrogen bonds, was conceptually inferred from the early theories of liquid water and aqueous solutions (Frank 1958; Kavanaugh 1964), and first demonstrated by early ab-initio quantum mechanical calculations on water complexes (Del Bene & Pople 1970; Hankins et al 1970). Cooperative patterns were found in the first systematic studies of hydrogen bonding in carbohydrates (Jeffrey et al 1977). The water molecule acting as acceptor in a particular hydrogen bond will strengthen the other hydrogen bonds of the water molecule acting as a donor (Tombari et al 1999), and so in liquid water cooperativity strengthens the hydrogen bonds by up to 2.5 times the strength of the hydrogen bond in the water dimer (Luck 1998).

This effect is also apparent in rings of hydrogen-bonded water. For example in the five-membered rings shown in Figure 1 the homodromic system (a) where the hydrogens all point in the same direction (clockwise or anticlockwise) around the ring is more stable than the antidromic system (b) where some hydrogens point in the same direction, which is itself more stable than the heterodromic arrangement (c) with only two hydrogens aligned (Peeters 1995).

Pure water in the solid state

Much more information can be obtained by diffraction methods on the structure of solids than of liquids. In a liquid there are continuous rearrangements of structure occurring within a very short time. This means that only a mean environment can be determined, which is the number and spatial arrangements of nearest neighbours of a molecule averaged over both time and space. The only information obtainable by X-ray diffraction of a liquid at a given temperature is the radial distribution function. This is the probability of finding an atom at a radius, r, from the central atom, averaged over both space and time. For example, an X-ray diffraction study on water will yield only the probability of finding an oxygen atom at a certain distance from another oxygen.

With the advent of neutron diffraction, using deuterated samples, distribution factors can be determined which give the O...O, O...D and D...D spacings from which the hydrogen-bonding patterns in the first hydration shell can be inferred, but interpretation of these patterns in terms of nearest neighbours is very difficult.

On the other hand, X-ray diffraction on a solid sample will usually establish the crystal structure (i.e., will yield the coordinates of all non-hydrogen atoms). Using neutron diffraction techniques with deuterated samples, the positions of the hydrogens (deuterous) can also be found, allowing hydrogen-bonding patterns to be clearly and unequivocally elucidated in the solid state.

Ices

The ice structures reveal the structural patterns for the regular cohesion of water molecules in the absence of other species. Arrangements of four-connected tetrahedrally or nearly tetrahedrally coordinated hydrogen-bonded water molecules are found in all known ice structures. These ice structures are characterised by maximisation of the number of hydrogen bonds and minimisation of the short-range repulsive restraints.

At least 14 distinct ice structures exist, along with at least two distinct amorphous phases (Jeffrey & Saenger 1991). In all known ice structures each water molecule is hydrogen bonded to four neighbouring water molecules. Only ices Ih, III, V, VI and VII can be in equilibrium with liquid water. All other ices are not stable with liquid water under any conditions.

The high-pressure phase lines of ice-X and ice-XI are still subject to experimental verification. Two different forms of

ice-XI have been found, the high-pressure form having the hydrogen atoms equally spaced between the oxygen atoms (Benoit et al 1996) whereas the low-temperature form has ordered hydrogen bonding (Lobban et al 1998). Ices II, XIII, IX, X and XI (both forms) have ordered hydrogen bonding whereas in all other ices the hydrogen bonding is disordered even down to 0 K, if this is reachable (Suga 1997). As well as five- and six-membered water rings, the high-pressure ices contain four-, seven- and eight-membered rings (Saenger 1987).

It has been discovered that the structure of normal ice (Ih) is not as simple as originally thought. To explain two well-separated optic bands in a high-resolution inelastic neutron-scattering study of ice, two different interaction strengths of hydrogen bonds were proposed (Li & Ross 1993). It was estimated that the numerical ratio of strong-to-weak hydrogen bonds was of the order of 2:1. The two interaction strengths were attributed to dipole or other types of electronic interactions, caused by proton disorder and the relative strengths were estimated to be approximately 2:1 (Li et al 1994).

It has recently been found that water under extreme pressure at low temperature (493 atmospheres at −40°C), between two hydrophobic plates held a nanometre apart, will contract into a two-dimensional glass rather than expanding into a known ice form. This glass, known as Nebraska ice, is made up of approximately planar 5-, 6- and 7-membered rings (Koga et al 2000).

Hydrogen-bond geometry in water in the solid state

A wide-ranging survey of all the known ice structures, together with data for clathrates and simple hydrates, found that the length of the hydrogen bond is remarkably constant in all the ices and differs only slightly with a wide variety of hydrogen-bond donors and acceptors (Plumridge et al 2000). The hydrogen-bond angle normally assumes values in the range $180 \pm 25°$, consistent with maintenance of the O...O distance (Figure 2).

Pure water in the liquid state

While there is general agreement that the structure of liquid water is determined by hydrogen bonding, the structure has been notoriously difficult to describe. In liquids, atom topologies exist for very short lifetimes, so they can only be observed by techniques recording the structure on an even shorter timescale. The techniques available, in decreasing timescale resolution, are infrared and Raman spectroscopy, dielectrical absorption and NMR spectroscopy, and inelastic neutron scattering. Despite the increasing scope of these techniques, for example by using isotopic substitution to enhance neutron diffraction data, the very short lifetimes of particular liquid-phase topologies, estimated on the basis of relaxation times to be of the order of 10^{-12} s (Frank & Evans 1945), mean that the only structural information which can be obtained by any of these techniques has to be averaged over time. Thermodynamic properties can be derived from the averaged long-lifetime atom distributions obtained from X-ray and neutron scattering.

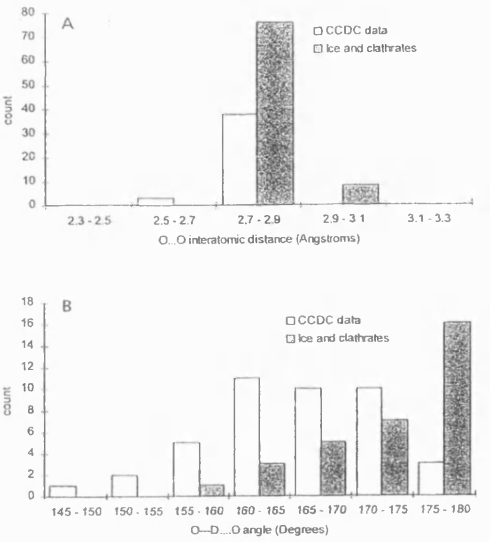

Figure 2 Survey of hydrogen-bond geometries in ices and clathrate hydrates, and small biological molecules from the CCDC. A. Distribution of O...O interatomic distances for ices and clathrate hydrates (grey bars) and hydrates of small biological molecules from the CCDC (clear bars). B. Distribution of O—D...O bond angle for ices and clathrate hydrates (grey bars) and hydrates of small biological molecules from the CCDC (clear bars).

In liquids, the molecules are in constant translational motion. Although this requires that long-range order is lost, there is still some short-range order. This short-range order has been found to extend to at least 8 Å even with totally inert, non-polar and spherically symmetrical molecules such as in liquid argon (Eisenstein & Gingrich 1942). The distance over which this short-range order exists should be greater when there is extensive hydrogen bonding, as in water.

There is a large number of anomalies in the physicochemical properties of water, including its unusually high melting point and boiling point, and the density anomaly. Whereas most liquids are less dense than their respective solids, water at 4°C is 8% more dense than ice. Upon melting, ice undergoes an increase in density that continues during heating until the temperature reaches 4°C. This contradicts the idea of thermal expansion, where most substances increase in volume with an increase in temperature. Above this maximum density, water behaves normally, decreasing in density with increasing temperature.

There are of the order of 38 such anomalies in the macroscopic properties of water and, while the physical bases of most are well understood, it has not been possible so far to devise a computer simulation method that can explain all of them.

Computer simulation of pure water

Since about 1980, increasing computing power has meant that complex simulations involving many hundreds of simulated water molecules have become possible. A large number of different models have been, and still are, proposed to represent water, and although each is capable of reproducing some of the macroscopic properties of water, no one model has explained all of them. Simulations involving just water molecules are now usually only run to test the ability of new water models to predict measurable thermodynamic properties of water and hence to refine the parameters involved in the simulation.

The models are usually used in conjunction with either molecular dynamics or Monte Carlo techniques, usually modelling dilute aqueous solutions involving of the order of a few hundred water molecules and usually only one or two solute molecules. These techniques are very powerful and are now routinely applied to increasingly complex systems such as docking ligands to hydrated protein structures.

Monte Carlo methods can be loosely described as statistical simulation methods where the simulation is driven by random numbers. Many simulations are performed (multiple trials or histories) using these random numbers and the desired result is taken as an average over the number of observations. In many practical applications, the statistical error can be predicted in this average result, and hence the number of Monte Carlo trials that are needed to achieve a given error can also be estimated.

The first Monte Carlo calculations on water that were able to simulate experimentally derived distribution functions with any degree of accuracy used 343 water molecules at 26°C (Ben-Naim 1971) using an improved Hartree-Fock potential with empirical corrections for the electron correlation energy. This success prompted Clementi and coworkers to lead a long and computationally expensive series of simulations beginning with the wave functions of water monomer, dimer, trimer and tetramer systems using potential functions from quantum mechanics, and Monte Carlo techniques to move the molecules classically (Clementi et al 1976). This study proved that accurate thermodynamic data could be obtained from ab-initio calculations in quantum chemistry (see Ab-initio section). At this time, no evidence from Monte Carlo studies had been found to support two-state or iceberg theories of liquid water.

Molecular dynamics simulation is a technique whereby successive configurations of the system are generated by integrating Newton's laws of motion, based on the positions and the velocities of the individual particles. In pictorial terms, atoms will move in the computer, bumping into each other, wandering around (if the system is fluid), oscillating in waves in concert with their neighbours, perhaps evaporating away from the system if there is a free surface, and so on, similarly to the way atoms in a real substance behave. This method allows the prediction of the static and dynamic properties of substances directly from the underlying interactions between the molecules.

Computer simulations have increased the demand for accuracy of the models. For instance, a molecular dynamics simulation allows the melting temperature of a material to be evaluated, and modelled with an interaction law. This is a difficult test for the theoretical model to pass so the simulation discloses critical areas and provides suggestions to improve the models.

When computer simulation results can be compared directly with experimental results, simulation becomes an extremely powerful tool, not only to understand and interpret the experiments at the microscopic level, but also to study regions which are not accessible experimentally, or which would need very expensive experiments.

Molecular mechanics studies of conformational preferences

Molecular mechanics treats molecules as atoms linked together with springs, allowing for harmonic bond stretching and bond angle bending. Each atom has finite spherical volume and relatively sharp boundaries with sinusoidal torsional energies. The force field for a typical molecule (e.g. a protein) can be given as a sum of the various components including bond stretching and bending, torsional potentials and non-bonded interactions. The resulting equations for the total energy can be combined with a variety of algorithms to locate energy minima on a conformational surface.

Models used to describe water

The TIP (transferable intermolecular potential) family of potentials has been used for modelling liquid water. TIP4P (Table 1) is a rigid four-site water molecule (Jorgensen et al 1983). This model attempts to capture the characteristics of a dipolar molecule by creating an imaginary fourth charged site on the bisector between the two hydrogen sites (Figure

Table 1 A comparison of common water models.

Model	Type	ε (kJ mol^{-1})	l_1 (Å)	l_2 (Å)	q_1	q_2	$\theta°$	$\Phi°$
SPC	A	0.6500	1.0000	–	+0.410	−0.8200	109.47	–
SPC/E	A	0.6500	1.0000	–	+0.4238	−0.8476	109.47	–
TIP3P	A	0.6364	0.9572	–	+0.4170	−0.8340	104.52	–
TIP4P	B	0.6480	0.9572	0.15	+0.5200	−1.0400	104.52	52.26
TIP5P	C	0.6694	0.9572	0.70	+0.2410	−0.2410	104.52	109.47

The type refers to the structures shown in Figure 7 (Jorgensen et al 1983; Mahoney & Jorgensen 2000).

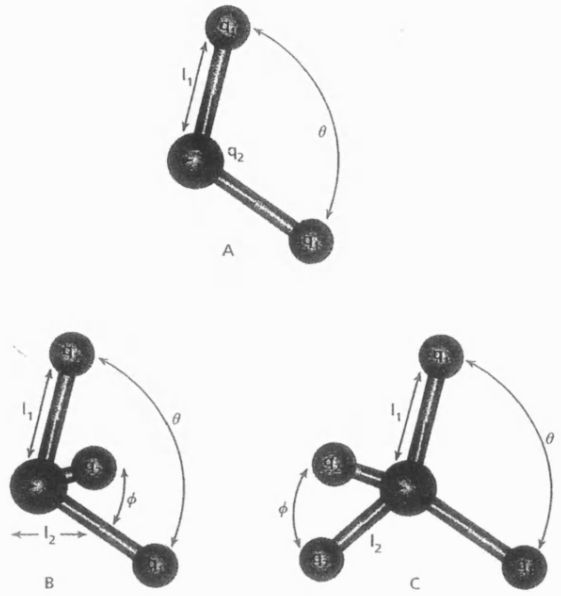

Figure 3 Models used to describe water.

3). The imaginary fourth site and the two hydrogens have an electrostatic charge, while the oxygen has only a Lennard-Jones potential. The parameters were obtained by fitting experimental, thermodynamic and X-ray structural data for liquid water at 298 K and 1 atm.

This model yields reasonable geometric and energetic results for the water dimer and is routinely used in simulations of aqueous solution (Barnes & Finney 1979).

SPC, the simple point charge model (Table 1), is an intermolecular potential function that gives good agreement with liquid water. This is a three-point charge model with a (6–12) Lennard-Jones potential on the oxygen, and charges of 0.41 on the hydrogens and −0.82 on the oxygen. The water geometry is shown in Figure 3, with O—H distances of 0.9572 Å, and an H—O—H angle of 104.52°. SPC/E is an extended version of this model.

Recently, the TIP5P model has been developed (Mahoney & Jorgensen 2000). This is a variation on the TIP3 and TIP4 families of models with five atom centres (i.e., the lone pairs are explicitly defined). Although this is a new model that has not yet been used widely, in-house development has shown that this model is more accurate than any previous simple models. The TIP5P model accurately predicts the diffusion constant of water at temperatures between −25 and 75°C and pressures between 1 and 300 atm. The predicted value is 2.62 ± 0.04 as compared with the experimental value of 2.30 (Mahoney & Jorgensen 2001). This is much better than any other method, with TIP4P predicting 3.29 ± 0.05, and significantly improves the model's ability to describe the maximum density anomaly.

The water models are tested by how accurately they can reproduce quantitative information that can be determined experimentally. Table 2 shows that recent models can predict the physical properties of water with a reasonable degree of accuracy. However, the approximations involved mean that no present model can explain all the macroscopic properties of water (Finney 2001).

These and other similar models are routinely used in conjunction with either molecular dynamics or Monte Carlo simulation techniques to predict low energy configurations of aqueous solutions, or using ab-initio techniques if the system is simple enough to be solved by this method.

Ab-initio calculations

Ab-initio molecular orbital computation has reached the stage that in some areas the calculations are more accurate than experimental results. However, there are so many possible variables in terms of the position of the nuclei that even in a system such as $(H_2O)_3$ a vast amount of computer time is required to satisfy the statistical mechanics constraints.

It is possible to calculate extremely accurate wave functions for monomeric H_2O incorporating the effects of many thousands of excited configurations. Properties such as dipole moment, charge distribution and spectroscopic

Table 2 Comparison of physical properties of water calculated for each of the common water models with experimentally determined results.

Model	Dipole moment	Dielectric constant	Self diffusion (10^{-5} cm^2 s^{-1})	Average configurational energy (kJ mol^{-1})	Density maximum (°C)	Expansion coefficient (10^{-4}°C^{-1})
SPC	2.27	65	3.85	−41.0		
SPC/E	2.35	71	2.49	−41.5	−38	
TIP3P	2.35	82	5.19	−41.1	−13	9.2
TIP4P	2.18	53[a]	3.29	−41.8	−25	4.4
TIP5P	2.29	81.5	2.62	−41.3	+4	6.3
Expt	2.65, 3.0	78.4	2.30	−41.5	+3.984	2.53

Expt = experimentally determined. All the data are at 25°C and 1 atm, except [a]at 20°C (Jorgensen et al 1983; Kusalik & Svishchev 1994; Mahoney & Jorgensen 2000).

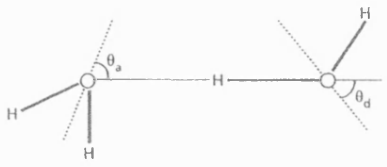

Figure 4 The structure of the water dimer. $R_{OO} = 2.98$ Å; $\theta_a = 58°$; $\theta_d = 50°$ (Dyke et al 1977). Dotted lines are the bisectors of the H—O···H angles.

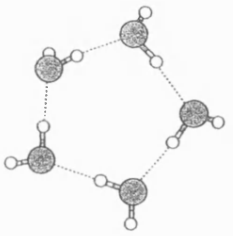

Figure 5 The global minimum conformation for five TIP4P molecules (Wales & Hodges 1998).

constants have been calculated many times in good agreement with experimentally determined values (see for example Goddard & Hunt 1974), but the techniques become much more computationally demanding when larger water complexes are considered.

The water dimer $(H_2O)_2$ has been studied spectroscopically (Dyke et al 1977) by expanding water vapour through a pinhole nozzle to produce a molecular beam of hydrogen-bonded dimers. Radio frequency and microwave transitions were observed, resulting from changes in the rotational energy of the dimer. These spectra were interpreted along with the dipole moment of the dimer to give the structure in Figure 4. This is the trans-linear complex and is in reasonable agreement with previous ab-initio calculations (Morokuma & Pedersen 1968; Lie & Clementi 1975), with the intermolecular distance in excellent agreement, good agreement with θ_d but only poor accord with the θ_a angle. Such comparisons between theoretically calculated structures without prior structural knowledge and experimentally determined structures allow models to be evaluated, and indeed many of the currently used models for liquid water are derived from potential functions tested on the water dimer. The best fit with the water dimer was given by a simple point charge model, similar to those proposed by Bernal & Fowler (1933).

The calculations become much more complex when considering three or more waters. The water trimer was investigated regularly in the 1970s and '80s (e.g. Del Bene 1971), but the structure remained unresolved; the investigators could not be certain whether it was an open structure with two hydrogen bonds or a cyclic structure with three. McDonald & Klein (1978) concluded that there was little chance of reconciling the properties of the liquid and gaseous states on the basis of a single water dimer potential. The first accurate calculations on the structures of clusters involving three and four water molecules were carried out by Clementi and coworkers (Niesar et al 1990). They found that the extra waters make the clusters more stable than the dimer. Model potentials were derived which included many-body terms and when used in simulation of bulk water gave radial distribution functions that agreed well with those deduced from X-ray and neutron diffraction studies. Calculations using the best currently available potentials have indicated that a square ring of four water molecules and a cubic array of eight waters are particularly stable (Vegiri & Farantos 1993). Cooperative effects have also been demonstrated by ab-initio computation. It has been shown that a cyclic homodromic water tetramer has 30% more energy than four isolated dimers (Koehler et al 1987).

Various quantum mechanical calculations on randomly generated arrangements of water molecules have shown that rings are more stable than chains (Gregory & Clary 1996), and it has been experimentally verified that the predicted minimum energy structures are found in bulk water (Liu et al 1996). Figure 5 shows the global minimum energy conformation for five TIP4P water molecules.

Weinhold (1998a) has used a combination of ab-initio

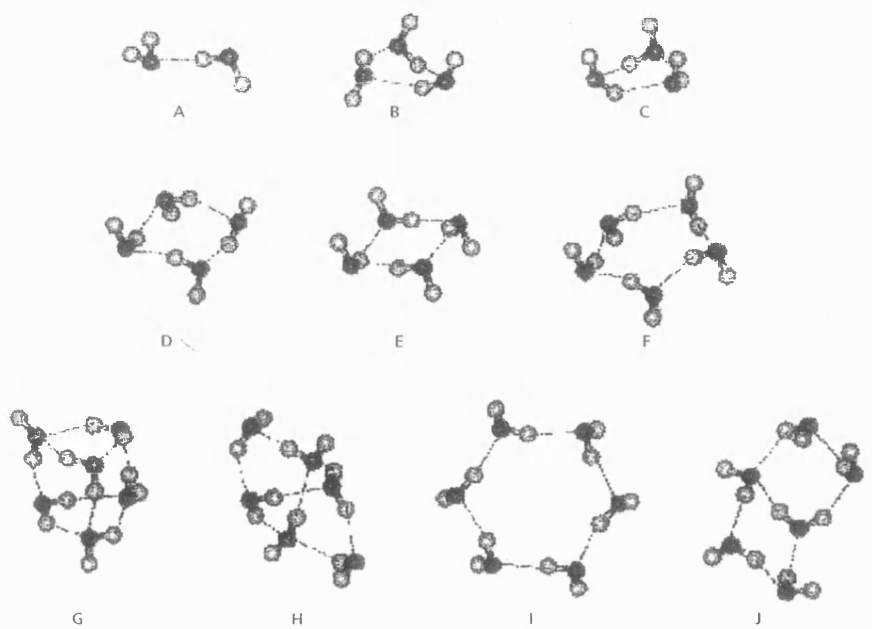

Figure 6 Geometry configurations, corresponding to the stationary points on the potential energy surface of the $(H_2O)_n$ clusters as predicted by the diatomics-in-molecules (DIM) method (reprinted from Grigorenko et al (2000) with permission from the American Institute of Physics and Dr Grigorenko).

computational techniques and Natural Bond Order analysis to shed light onto the electronic principles governing hydrogen-bonded clusters. The energetics of these clusters were found to be largely dependent on cooperative charge transfer which led to aggregation patterns markedly different from those predicted by the pairwise-additive potentials in common usage. Weinhold devised the quantum cluster equilibrium theory of hydrogen-bonded liquids in which the clusters are treated as the fundamental constituent units rather than individual water molecules. This method has been applied to water and has demonstrated numerical accuracy for a wide range of thermodynamic properties (Weinhold 1998b).

A related method by which potential energy surfaces of hydrogen-bonded aggregates are generated, the diatomics-in-molecules (DIM) theory has been developed to overcome some of the shortcomings of traditional molecular mechanics techniques using quantum mechanical calculations. The technique was applied to hydrogen fluoride clusters (Nemukhin et al 2000) and was found to give results in good agreement with both experimental information and previous simulations. The theory has been applied to water clusters of up to six molecules (Grigorenko et al 2000) and the minimum energy structures are shown in Figure 6.

This method is very computationally demanding, but the structures and binding energies of the complexes show a better correlation with experimentally derived terms than has previously been shown with any simulation method.

Various novel computational methods have been devised that bypass the conventional water models and methods. Chaplin (2000) attempted to explain water structure in terms of a network constructed from icosahedral water clusters. The 14-water structures (see Figure 7) can be joined together to form large networks. This model can explain some, though not all, of the properties of liquid water.

A further approach used the geometric information described in Figure 2 to constrain a simulation of water molecules with various solutes in which hydrogen bonds would be accepted if their length and angle fell within stated boundaries (Plumridge et al 2000). The simulation has built large ice-like structures and successfully distinguished between structure-making and structure-breaking solutes. This approach assumes that the hydrogen-bonding observed in solid water is similar to that in the liquid state, an assumption that appears more likely as modern techniques reveal the details of liquid water structure. This simulation would not succeed with a continuum model of water structure.

Water in contact with interfaces

Thermal expansion of water in glass capillaries shows normal behaviour in tubes of inner diameter greater than

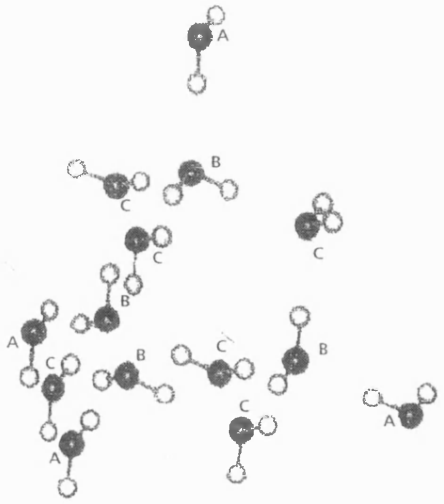

Figure 7 Icosahedral water cluster (reprinted from Chaplin (2000) with permission from Elsevier Science and Prof. Chaplin).

1 μm, but in narrower tubes the curves began to deviate. In fine capillaries the minimum specific volume at 4°C disappears and the coefficient of expansion becomes constant (Fedyakin 1962). Metsik studied the thermal conductivity of water between mica sheets, and found normal conductivity in films wider than 1 μm (Derjaguin 1970). However, as the film becomes thinner the conductivity rises sharply. At a thickness of 0.1 μm, the thermal conductivity is ten times that of normal water (Derjaguin 1970). It is worth noting that a 1-μm film is more than 3000 water molecules thick.

Hori (1956) measured the freezing point of water between mica sheets and polished glass surfaces. He found that if the film is between 1 mm and 10 μm, the freezing point is between -10 and -30°C. At a thickness less than 10 μm, he found no evidence of freezing down to -90°C. Hori also studied the equilibrium vapour pressure of thin water films held between a flat and a curved glass or quartz surface. When the film was thicker than 1 μm, the vapour pressure decreased with increasing temperature. Below this the vapour pressure was lower than that of the thicker film at the same temperature, and when the films were as thin as 0.1 μm there was no detectable vapour pressure at temperatures up to 300°C.

DeBoer & Zwikker (1929), in an early study of the condensation of water on glass surfaces, presented a theory whereby multi-layer condensation was attributed to a propagation of the electric polarisation at the polar solid surface. They also found that thicker layers built up if the solid surface contained alternating positive and negative charges. Ling (1971) found that if a surface contains positive and negative charges in a regular array, and in particular if two of these surfaces are brought together, then deep layers of water will exist in an ordered array of dipolar lattices. A stabilised three-dimensional matrix of polarised water molecules may then be established.

Soviet soil scientists have studied water in soils, and categorised it as either being suspended (i.e., similar to bulk), loosely bound or bound. Bound water is the liquid in air-dried soils, and is no more than one or two molecules thick. The loosely bound water has been described as being formed by a process of multilayer sorption by successive polarisation of water dipoles. The thickness of such envelopes may be hundreds or even thousands of molecular diameters. Loosely bound water has a diminished capacity for dissolving electrolytes. (Forslind & Jacobsson 1975). NMR studies by Ducros (1960) and Woessner & Snowden (1969) have shown signal splitting in the deuteron signal of D_2O–clay systems, indicating that the dipole–dipole interactions do not average out as in liquid water, indicating deep layers of structured water.

Ever since the discovery (Derjaguin 1966) and subsequent demise of polywater (Rousseau & Porto 1970), most novel work on water has been viewed very sceptically by the scientific community. In fact, the analyses which disproved the polywater findings were also found to be flawed. The samples were only available in miniscule volumes and there were delays in the analysis. Some samples had no impurities, and some samples contained silica that was thought to be insoluble in water. An unrelated high-density form of liquid water has since been proved to exist at low temperatures (Mishima & Stanley 1998).

It is only recently that neutron diffraction and spectroscopic techniques have reached high enough resolutions to investigate pure water in confined conditions. A neutron diffraction study of water confined in Vycor glass, with pores of 40 Å on average, showed that the orientational preferences of the confined water molecules are very different from those of bulk water (Bruni et al 1998). Molecular dynamics using data from this experiment predict that the waters have very slow relaxation times, and this was thought to indicate the presence of longer-lived cavity structures encaging other waters (Starr et al 1999).

Recently, several groups have investigated water in confined conditions by means of ultrafast laser spectroscopy to shed light on dielectric relaxation times (Bhattacharyya & Bagchi 2000). The most striking of the results is that water, in a variety of conditions, exhibits a bimodal response, with not only a normal fast sub-picosecond response as would be expected from bulk water, but also a slow component in the timescale between hundreds and thousands of picoseconds. This slow component constitutes 10–30% of the total response and is totally absent in pure water. The confined environments in these studies were a range of molecular assemblies, such as reverse micelles and microemulsions (Riter et al 1998), cyclodextrin (Vajda et al 1995), micelles (Telgmann & Kaatze 2000), lipids (Datta et al 1998), proteins (Jordanides et al 1999) and DNA (Halle & Denisov 1998), as well as several macroscopic solids which trap water (e.g. hydrogels (Datta et al 1997)). In all cases, the slow component was detected and, after discussion between the groups, its origin was attributed to a

dynamic exchange between free and bound water. A comprehensive understanding of the mechanism and implications of this phenomenon is not yet available.

Overall, it is apparent that water in contact with regular hydrogen-bonding surfaces can adopt the pattern expressed at the surface and extend that pattern out into the bulk water, at least to several hundred molecular diameters. Such well-characterised effects are reminiscent of the early ideas on cellular water (see above). It would be ironic if the very earliest ideas on cellular water were found to have merit, nearly 150 years after they were first expressed.

Aqueous solutions

Solid solutions

The advantage of studies on solids is that all the intra- and inter-molecular parameters can be measured with confidence. While extrapolation to liquids must be approached with caution, some valuable conclusions can be drawn concerning the intrinsic properties of aggregates of water molecules. These aggregates may be present as simple or complex hydrates, or clathrates. There is increasing evidence for the formation of comparable structures in liquid water.

Hydrate inclusion compounds

Hydrate inclusion compounds are solid crystalline complexes in which water molecules are hydrogen bonded to form regular three-dimensional four-connected networks. Guest molecules are included either in void spaces in the network, or hydrogen bonded to the water network. At present, four classes of hydrate inclusion compounds are known: clathrate hydrates, alkylamine hydrates, alkylammonium salt hydrates and the polyhydrates of some strong acids.

Clathrate hydrates are a type of hydrate inclusion compound in which a small, usually non-polar, guest molecule is encaged in a host network of hydrogen-bonded water. The guest in true clathrate hydrates will only interact with the host through van der Waals interactions with no other guest–host bonding involved. In the alkylamine hydrates, hydrogen bonds are formed between the amine group and the water network. Alkylammonium salt hydrates are formed with an anionic guest inside a cationic host lattice.

The first reported preparations of a crystalline clathrate hydrate were made by Davy in 1811 and then by Faraday in 1823, who were both investigating the hydrate of chlorine. The term clathrate was introduced by Powell (1948), who deduced that these were, in fact, inclusion compounds and named them from the Greek for lattice. Concurrent work by three teams, von Stackleburg & Muller (1951), Pauling & Marsh (1952), and Claussen (1951a, b) established the common 12-Å and 17-Å clathrate structures. These structures, now referred to as structures I and II, accounted for the stoichiometry and powder diffraction patterns for the majority of known hydrates. The most common clathrate cavity types are shown in Figure 8. Structure-I clathrates are comprised of pentagonal dodecahedra and tetrakaidecahedra (A, B) while structure-II clathrates are built from pentagonal dodecahedra and hexakaidecahedra (A, C). The pentagonal dodecahedron corresponding to $(H_2O)_{20}$ occurs in all true clathrate structures and many related inclusion compounds.

Clathrates are known to occur in natural environments, from deep-sea sediments (Appenzeller 1991) to nucleation processes in the atmosphere (Yang & Castleman 1991). Upon decomposition they can cause explosions in high-pressure gas pipelines (Kelkar et al 1998).

A clathrate of CH_3CCl_2F (CFC R-141b) has been observed to form at the interface between liquid water and liquid CH_3CCl_2F (Ohmura et al 1999). The system was observed with a video camera with a micrographic zoom lens, and plate-like crystals were formed over a number of hours, that dissolved if the temperature was raised. The water shell was apparently left unchanged, as the crystals reformed if the temperature was lowered again.

A series of alkylammonium salt hydrates were first prepared by Fowler and co-workers in 1940 (Fowler et al 1940). Unlike the clathrates, each guest molecule produced a different host water lattice. At the request of Henry Frank, McMullan & Jeffrey (1959) carried out an initial X-ray diffraction study of these compounds. This work revealed structures very similar to the clathrates and a subsequent detailed study showed close structural

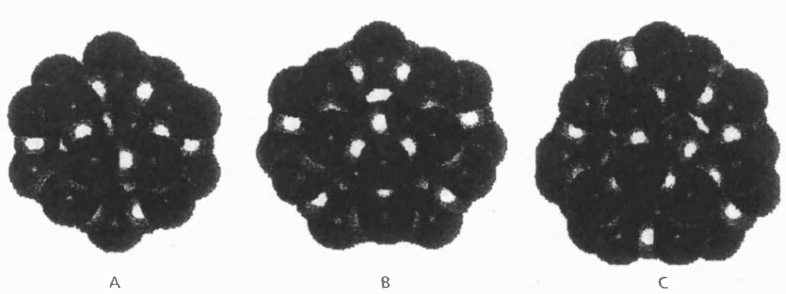

Figure 8 The cavity types in structure-I and -II clathrate hydrates. A. Pentagonal dodecahedron $(H_2O)_{20}$. B. Tetrakaidecahedron $(H_2O)_{24}$. C. Hexakaidecahedron $(H_2O)_{26}$.

analogies, with some alkylammonium salt hydrates being isostructural with clathrate hydrates.

Jeffrey and co-workers carried out similar diffraction studies of alkylamine hydrates, which again had different lattice structures for each guest molecule (McMullan et al 1966). These structures included hydrogen bonding between the guest and the water lattice, but again there were many similarities with the clathrates. Using results from these three types of compound, a common structural theme was discovered, based on the $(H_2O)_{20}$ pentagonal dodecahedron.

Some strong acids (HPF_6, HBF_6 and $HClO_4$) were found to form crystalline hydrates at lowered temperatures, and X-ray diffraction confirmed that they were iso-structural with the 12-Å cubic structure-I clathrate hydrates. These were the first clathrate structures where cationic hosts were found (Mootz et al 1987).

In inorganic hydrates, the water molecules generally complete the coordination shell of the cations, and hydrogen bond to the anions. This means that the structures formed are governed largely by the stereochemical requirements of the ions rather than those of water.

Organic hydrates can be subdivided into hydrates with isolated water molecules, in which the structure is determined principally by the ionic coordination or hydrogen-bonding function of the solute, and water framework structures. Water framework structures include the hydrate inclusion compounds, and also systems in which water molecules form nets, sheets, columns, ribbons or chains, with the functional groups of the organic molecules strongly influencing the structure, and no recognisable clathrate voids (Luck 1998).

The three-dimensional structures of all biological macromolecules are intimately associated with water. The solid-state hydration structures of small biological molecules (carbohydrates, purines, pyrimidines, nucleosides and nucleotides) are determined mainly by packing forces, and hydrogen bonding between the functional groups of the organic molecules. Water plays a secondary role in these structures, occupying the space between the organics, and adding to the hydrogen bond energy of the lattice while in competition with the molecular packing in the absence of water. If biological molecules aggregate, or if a substrate enters the active site of an enzyme, the water molecules have to move from the contact surface of the biological molecule in a coordinated manner with the least expenditure of energy. Hydrogen bonding must play an important role in this substitution process (Jeffrey & Saenger 1991).

Aqueous solutions in the liquid state

As has been stated above, the structural information that can be obtained from diffraction studies on liquid species is limited because of the continual translational and rotational movement of the molecules. However, the increasing sophistication of computer simulation techniques along with accurate models of the water molecule has allowed the structural aspects of aqueous solutions to be investigated.

Computer simulations

Molecular dynamics studies on aqueous solutions Molecular dynamics has been used to study the hydrophobic hydration of noble gases (Tanaka & Nakanishi 1991). It was found that the introduction of a xenon solute gives rise to a restructuring of the water to form a clathrate-type structure. This is shown by an increase in population of the cyclic water pentamer, which gives rise to an exothermic hydration process.

A molecular dynamics simulation (Skipper 1993) of a system containing four methane molecules and 256 waters at around room temperature found solvent-separated methane interactions which were compatible with a first hydration shell in which the water dipole vectors were predominantly tangential, and a second hydration shell where they were predominantly radial. This orientational information and the sizes of the cavities are compatible with a simple clathrate-type structure. A similar, though more exhaustive and computationally more complex, study (Marcera et al 1997) confirmed these findings.

Molecular dynamics has also been used to study the hydrogen-bonded network in liquid water. In a study of liquid water, topological defects were found which directly affected the mobility of the water. The network defects act as catalysts, providing lower energy pathways between different tetrahedral local arrangements (Sciortino et al 1992).

Molecular dynamics has been used to investigate water clusters. $(H_2O)_n$ clusters, where $n = 3$–6, were investigated by Dang & Chang (1997). They found cyclic planar structures to be the most stable for $n = 3$–5. For the water hexamer, several structures were suggested, very close together in energy. A prism-like structure was found to be the lowest energy structure, with cyclic boat and cage structures at slightly higher energy. Using the same methods, Dang (1999) characterised the water octamer, nonamer and decamer, and investigated the water–iodide cluster. The lowest energy octamer was found to be a rough cubic structure with four-membered rings, the nonamer was a similar system with a five-membered ring, and the decamer was found to be a system with two five-membered rings one above the other with hydrogen bonding between the rings. The iodide–water clusters are in excellent agreement with optimised structures developed from accurate electronic structure theory calculations (Combariza et al 1993).

Molecular dynamics has been used together with a number of novel water potentials (van Maaren & van der Spoel 2001) designed to investigate biomolecular hydration. Flexible, as well as fixed, water models were tested and it was found that varying the water potentials based on their distance from the biomolecule improves the accuracy of the method to reproduce measurable liquid-phase properties. This work, though still in its early stages, could help to elucidate the role of water in biological processes.

Another water potential has been devised to reproduce long-range effects by putting diffuse charges on the hydrogens and oxygens as well as the normal point charges (Guillot & Guissani 2001). This method can accurately

model polarisation effects when calculating the dipole moment, and so the technique can accurately predict the dielectric constant at any state point. This supports the idea that induction effects in water are more important than previously expected.

Monte Carlo simulations of aqueous solutions. The first Monte Carlo simulations of aqueous solutions were reported for the systems Li^+F^- and Li^+Cl^- (Fromm et al 1975) and Na^+F^- and K^+F^- (Clementi et al 1976). The coordination and shell radii were found to be in good agreement with neutron diffraction experiments.

The first Monte Carlo simulation claiming to reproduce the iceberg model was carried out on pure water and an infinitely dilute solution of methanol (Nakanishi et al 1981). Water molecules in the vicinity of the hydrophobic methyl group are energetically more stable than those in the hydrophilic region. This is clear evidence of conventional hydrophobic hydration where a water cage structure forms around the hydrophobic group.

A study of the aqueous hydration of benzene has shown a complex with two water molecules attached, one either side of the π-cloud, and a hydration shell with 21 water molecules around this (Ravishanker et al 1984). The structure involves puckered 5-membered rings of waters, and also larger ring structures. Water–π interactions have been observed in aqueous benzene using X-ray diffraction (Atwood et al 1991). This system has been re-investigated (Urahata & Canuto 1999) with more advanced techniques. The results confirmed these findings, the authors concluding that the first hydration shell must be similar to a clathrate cavity.

In a similar study of the hydration of small alkanes, the hydration numbers in the first hydration shell were determined using a TIP4P model of water together with an OPLS set of Lennard-Jones parameters optimised for liquid hydrocarbons (Jorgensen et al 1985). The hydration shells were consistent with clathrate structures with more irregular forms. Methane formed a structure-I clathrate with an $(H_2O)_{20}$ cavity in close agreement with the Monte Carlo value of $(H_2O)_{20.3}$ (Jeffrey & Saenger 1991).

A study of the hydration of ethanol with TIP3P waters using Monte Carlo techniques predicted the hydration energy of -57.1 ± 6.5 kJ mol^{-1}, agreeing well with the experimental value of -54 kJ mol^{-1} (Levchuk et al 1991). This meant that more complex problems could be tackled. In an attempt to understand the interactions between protein denaturants and aromatic rings, the approach of urea and guanidinium to benzene, and the approach of urea to naphthalene in aqueous solution were studied by Monte Carlo techniques (Duffy et al 1993). Single minima were observed at around 4 Å in all cases, which indicated the existence of direct interactions between the structure breaker and the aromatic ring with unstructured water in the vicinity.

Recent Monte Carlo studies have shown that water structuring around hydrophobic solutes is very sensitive to solute size and shape (Fang et al 1997). The hydration structure around hypothetical spherical ions was shown to generate clathrate-type structures if the radius of the ion was within specific limits, whereas different sized and shaped ions produced much more irregular structures. A similar investigation (Martinez et al 1999) showed that, when considering small highly charged ions such as Cr^{3+}, it is more useful to consider the hydrated ion's interactions with bulk water rather than the naked ion (e.g. $[Cr(H_2O)_6]^{3+}$ is used rather than Cr^{3+}). This has been shown to reproduce experimental radial distribution functions more accurately.

A Monte Carlo study of the hydration of methane (Hernandez-Cobos et al 2001) gave essentially the same results as the molecular dynamics simulation (Skipper 1993), and the direct experimental methods (Bowron et al 1998c – see EXAFS spectroscopy section, below) with disordered hydration shells around the methane. The radial distribution functions from the simulation compared very favourably with those for aqueous methane (Soper & Finney 1993).

Advanced Monte Carlo techniques involving specialised potentials have been applied to the hydration of Li^+ (Lyubartsev et al 2001). The results show almost identical radial distribution functions to those derived from neutron diffraction (Howell & Neilson 1996) with four waters arranged tetrahedrally around the ion.

Reverse Monte Carlo (McGreevy 1990) is a computer simulation technique which works with scattering patterns derived from, for example, neutron diffraction or EXAFS patterns (see below). The process then generates configurations that will fit the radial distribution curve. No potentials are used, and the system works by optimising the difference between the experimental pattern and the pattern generated by the trial system. This has been applied to liquid water (da Silva et al 2001) and has successfully reproduced energy terms and hydrogen-bond configurations, but flaws in the existing water models have been highlighted in this study.

Experimental evidence for the structure of liquid aqueous solutions

X-ray and neutron diffraction. In an X-ray diffraction study of aqueous *t*-butanol at 20°C, Nishikawa & Iijima (1990) found that, at mole fractions around 0.17, some *t*-butanol molecules were encaged by water molecules in clusters, which tended to clump together in larger aggregates of structured water by sharing their polyhedra. They found that for compositions $(TBA)_m(H_2O)_l$ the ratio l/m is between 17 and 25, consistent with the structure-II clathrate structure. At a higher temperature (55°C) they found evidence of clusters, but at this temperature the structures were looser and more distorted.

In an attempt to understand the structure-breaking properties of nitrate and perchlorate ions, neutron diffraction experiments have been carried out on concentrated aqueous solutions using isotopic substitution on the chlorine. The results showed that both species have a reduced number of waters in the first hydration shell: 3–4 for NO_3^- (Neilson & Enderby 1982) and 4–5 for ClO_4^- (Neilson et al 1985) compared with, for example, the value

obtained for Cl^- of 7–8 (Neilson & Enderby 1983). The distances between the central atom and the deuteriums and oxygens of water are very similar in all three cases (carbon–deuterium distance = 2.9–3.1 Å; carbon–oxygen = 3.6–3.8 Å). This is a consequence of the strength of the structure-breaking properties, nitrate > perchlorate > chloride.

A more recent higher resolution neutron diffraction study on the hydration of chloride (Yamaguchi & Soper 1999), using a more advanced procedure to refine the data, found 9–10 waters in the first hydration shell, but found that only 5–6 pointed a deuterium at the chloride atom (i.e., were involved in conventional hydration).

The use of hydrogen/deuterium (H/D) isotopic substitution in structural studies of water and organic liquids provides more information, particularly when hydrogen bonding is involved (Soper & Egelstaff 1981). Separated partial distribution functions can be obtained from the radial distribution function when partially deuterated species (e.g. HDO) are used which can give direct information on local hydrogen-bond configuration. This technique can also be used to acquire more structural information when dealing with aqueous solutions (Turner et al 1990; Bowron et al 1998c).

A neutron diffraction experiment on a 1:9 molar ratio methanol–water mixture, in which isotopic substitution was used to isolate the methyl-hydrogen to water-hydrogen pair correlation function from the other interactions, confirmed the existence of a definite hydration shell of water molecules at a distance of 3.7 Å from the methyl carbon atom (Soper & Finney 1993). The water molecules in this shell formed a disordered cage but retained the roughly tetrahedral local coordination found in pure water. Some degree of polarisation of the water molecules was also apparent, and the shape of the shell implied a weak alignment of the water molecule dipoles with that of the methanol. This was the first experimental evidence of a hydration shell around methanol, and served to reinforce Frank's theories, as well as closely agreeing with the results of various simulation studies (Okazaki et al 1984; Ferrario et al 1990; Guillot et al 1991; Skipper 1993).

Neutron diffraction with isotopic substitution was used to study the hydration structure of methane in aqueous solution (De Jong et al 1997). H/D substitution was used on both the solute and the solvent to determine the partial pair correlation functions. Analysis of these functions showed that the methane molecule is surrounded by 19 ± 2 water molecules, and showed that these waters were tangentially oriented with respect to the methane. This figure is in excellent agreement with the pentagonal dodecahedron, with twenty waters found in clathrates and many simulations.

A recent study, on concentrated sodium hydroxide in the liquid state using neutron diffraction with isotopic substitution (Bruni et al 2001), showed that a large change in the water structure relative to that of pure water occurred at around 300 K. The peaks corresponding to orientational correlation between neighbouring water molecules were considerably shifted relative to those of pure water and this was thought to indicate that ions in aqueous solutions induce a change in water structure equivalent to the application of high pressure.

Dielectric measurements. It is known that the dielectric relaxation times of liquids are influenced by temperature and concentration. The relaxation times can be derived from time domain reflectometry in the microwave region (commonly 500 MHz to 25 GHz) at different temperatures and concentrations. In considering interactions in water mixtures, accurately measured dielectric relaxation times allow the activation enthalpy (ΔH) and entropy (ΔS) to be separated from the activation free energy (ΔG), and hence allow excess partial molar activation free energy, enthalpy and entropy to be calculated for the water and for the other component as a function of temperature and concentration. Maxima in the excess enthalpy and entropy are due to structural enhancement of the water network by the other component.

In a study of ethanol–water mixtures using these methods (Sato et al 1999), two maxima were found in both the excess enthalpy and entropy of ethanol in the dilute region. These maxima occurred at mole fractions of ethanol in water of 0.04 and 0.08 which were clearly attributed to structural enhancement of the water network by the ethanol. The mixing schemes for the two points appear to be qualitatively different. The 0.04 mole fraction corresponds to a ratio of one ethanol to 24 water molecules, possibly indicating a clathrate-type cage. The 0.08 mole fraction refers to a ratio of one ethanol molecule to 12 waters; the type of structural enhancement associated with this maximum is unknown. At mole fractions of ethanol as low as 0.005 (one ethanol to around 200 waters), positive values in the rates of change of the excess enthalpy and entropy indicate that ethanol–ethanol interaction through the water network is operative with each ethanol surrounded in a spherical fashion by waters. At a mole fraction of approximately 0.15, a minimum is observed in the excess enthalpy and entropy of ethanol and a maximum in those of water. This can be attributed to the formation of a structure-I clathrate hydrate of the formula $6C_2H_5OH.46H_2O$. This is also suggested by adiabatic compressibility results on the ethanol–water system (Onori 1988).

In a further study of methanol–water mixtures using the same techniques (Sato at al 2000), again two maxima were found in the methanol excess enthalpy and entropy in the dilute region, both attributed to structural enhancement of the water network.

Microwave dielectric relaxation measurements have been used to study water structure around larger molecules. In a study of polysaccharides, all molecules showed two dielectric relaxation peaks, except for glucose which only showed one (Mashimo et al 1992). One peak is due to the orientation of the water molecules whereas the other is due to the orientation of the saccharide molecules. Glucose can occupy a site in an ice lattice corresponding to the position of a six-membered water ring. From this it was inferred that glucose behaves in a similar manner in liquid water, with several hydrogen bonds between glucose and waters, and that the glucose can only reorient itself when the water structure is broken.

Similar studies have been carried out using a sample of DNA (Mashimo 1994). The sample was dissolved in a buffer and subjected to microwave dielectric measurements at different temperatures. The DNA solution exhibited two peaks at all temperatures, attributed to bound and bulk water. However, measurements between −15 and −65°C, where all bulk water was frozen, showed definite evidence of the participation of two types of bound water. Part of the water would freeze gradually in the temperature range −15 to −35°C, but some water would not freeze at −65°C. These phases were shown to be different from bulk water, and each other, so were designated as tightly bound and loosely bound.

NMR. Deuterium spin-lattice relaxation times were reported for undercooled (180–300 K) solutions of tetraalkylammonium ions (Bradl & Lang 1993). A comparison with undercooled alkali-metal halide solutions allowed the competing influence of coulombic, hydrophobic and hydrogen-bond interactions on the dynamic structure of the transient hydrogen-bonded network to be studied. Solvent dynamics were found to be closely related to the glass-forming tendency of undercooled Me_4N^+ and Pr_4N^+ solutions, and the clathrate-forming tendency of the Bu_4N^+ solutions.

The NMR rotational correlation times for heavy water molecules have been measured in aqueous solutions of benzene and phenol. The measurements were made by the integrated capillary method and showed that the correlation time for shell water molecules was 4 times as long in benzene as in phenol. Benzene is a known structure maker, while phenol is a structure breaker. The increased correlation time indicates that the hydrophobic hydration of benzene is associated with a remarkable rotational slowdown, or increase in local viscosity (Nakahara & Yoshimoto 1995).

In a similar study, the ^{17}O NMR spin-lattice relaxation times of the solvent water have been determined in aqueous solutions of nine alcohols and five diols at 25°C (Ishihara et al 1997). From these data, the coordination numbers and rotation correlation times compared with pure water have been estimated. The correlation times are inversely related to the alcohol's structure-breaking capability, so *t*-butanol provides the longest-lived water structure.

Fourier transform infrared spectroscopy. Fourier transform infrared (FTIR) spectroscopy can be used to give insight into the structural perturbations which occur when hydrophobic solutes are introduced into water by examining the vibrational transitions occurring in the solute-induced hydration shells. Infrared spectroscopy has been used to detect the vibrations of DOH molecules in the solvation shells of inorganic salts (Kristiansson et al 1988). By a calculated difference spectrum, where the spectrum of pure water is subtracted from that of a solute in water, the water-structuring capacity of solutes can be quantitatively compared. Shifts in absorbance intensity and frequency reflect changes in populations of water molecules with different hydrogen-bonding patterns (Lindgren & Tegenfeldt 1974). In a study of methane, ethane, propane, butane, pentane and hexane sulfonates, Hecht et al (1992) compared the difference plots obtained from the FTIR analysis, and found shifts in the vibrational intensity and frequency which were consistent with increased ordering of the water shell as the hydrocarbon length was increased. These results are consistent with theories of hydrophobic interactions as well as other experimental techniques such as excess molar heat capacity measurements (Muller 1990) and relative solubility in aqueous and non-aqueous solutions (Nozaki & Tanford 1971).

Heat capacity measurements. The analysis of heat capacity changes is of central importance in understanding the thermodynamics of protein folding, protein–protein binding, protein–nucleic acid binding and hydrophobic effects (e.g. Murphy & Freire 1992). The improved sensitivity and accuracy of calorimeters has provided much heat capacity data. Heat capacity is useful as it relates enthalpy, entropy and free energy to each other. Processes such as protein folding or unfolding produce large changes in heat capacity due to the desolvation of polar and nonpolar groups (Sturtevant 1977). The change in heat capacity involved on hydration is large and positive for nonpolar groups, and negative for polar groups (Marcus 1994). This is more useful than entropy and enthalpy measurements as these hydration processes can be either entropy- or enthalpy-driven. Computer simulation has suggested that heat capacity changes can be explained by non-polar solutes causing a concerted decrease in the hydrogen-bond length in the first hydration shell and polar solutes increasing the oxygen–oxygen distance, and causing the hydrogen bonds to bend (Sharp & Madan 1997).

HPLC. The molar free energy, enthalpy and entropy of transfer of a non-polar solute from a highly polar liquid (e.g. poly(dimethylsiloxane)) into water can be deduced from HPLC measurements (Shinoda et al 1987). In a study of toluene and ethylbenzene in water, Silveston & Kronberg (1989) found a large positive free energy when the two probes were transferred from the polymer liquid into water, along with a large negative entropy change. This was interpreted as a consequence of water structuring around the toluene and ethylbenzene molecules.

EXAFS spectroscopy. Recent work on the hydrophobic hydration of krypton, using extended X-ray absorption fine structure (EXAFS) spectroscopy (Filipponi et al 1997; Bowron et al 1998a), has shown that the liquid-to-solid transition produces a clathrate hydrate, indicating that Frank's structuring of liquid water by non-polar solutes may be very similar to clathrate hydrate structures.

The EXAFS probe is intrinsically short-ranged and element specific, so is ideal for comparing local structure in the solid and liquid phases due to its insensitivity to long-range order in the crystalline phase. In this case the structural signal was dominated by $Kr–O_{(water)}$ correlations.

The size of the liquid hydration shell was found to correlate very well with the small structure-II clathrate cage, but this cage was more loosely defined than in the solid state. The experimentally determined coordination numbers indicated that one water molecule was drawn into the 4.5–5.1 Å range to form the large clathrate cavity, although the number of water molecules in the 2.6–5.7 Å region remained constant at 22.5 in both states. This figure agrees very well with the values for structure-II clathrates, where the small cavity is composed of 20 waters and the large cavity 26.

In a further EXAFS study, the temperature dependence of the hydrophobic hydration shell was investigated for krypton and xenon in the range 277–368 K (Bowron et al 1998b). The experiment provided clear evidence that as the temperature is raised, systematic thermal perturbation leads to increased entropy, and the cage becomes less clearly defined.

The hydration of Ca^{2+} was investigated in a combined EXAFS, large angle X-ray scattering and molecular dynamics study (Jalilehvand et al 2001). The results accurately confirmed the radii of the first and second hydration shells at 2.46 and 4.58 Å, as well as the number of waters in the first shell (8). These findings agreed well with existing experimental data on the system and the molecular dynamics results.

Structured water in the gaseous state

Clusters are weakly bound aggregates of atoms or molecules. Water clusters occur in the gaseous phase and have been studied extensively to further an understanding of hydrogen bonding, and solvation and nucleation phenomena in general. In the mass spectra of polymeric compounds or complexes the appearance of prominent peaks in an otherwise continuous distribution of signals is called a magic number cluster, and may indicate the existence of species with enhanced stability.

In water systems, it is well known that the cluster corresponding to $(H_2O)_{21}H^+$ always exhibits a pronounced magic number under different experimental conditions (e.g. expansion of ionised vapour (Beuhler & Friedman 1982), ion bombardment of ice surfaces (Haberland 1984); electron impact ionisation (Echt et al 1989) and vacuum photoionisation of neutral clusters (Shinohara et al 1985)).

Shinohara et al (1985) employed a neutral supersonic nozzle linked to a molecular-beam mass spectrometer supplied with premixed water–ammonia gas, to investigate the formation of mixed binary water–ammonium clusters. Evidence was found for exceptional structural stability of protonated clusters corresponding to $(H_2O)_{20}(NH_3)_mH^+$ (m = 1–6) and $(H_2O)_{27}NH_4^+$. A parallel Monte Carlo simulation yielded larger binding energies for these structures compared with their close neighbours, in agreement with the mass spectrometry results, and a deformed pentagonal dodecahedron enclosing an NH_4^+ ion was proposed, with the stability due to strong coulombic interactions (ionic hydrogen bonding) between the NH_4^+ and the 20 waters, as well as the inherent stability of pentagonal rings and the pentagonal dodecahedron. The authors suggested that this may also explain the exceptional stability of the $(H_2O)_{21}H^+$ cluster. To account for the exceptional stability of this cluster, a pentagonal dodecahedral structure has been proposed (Miller et al 1983) in which H_3O^+ is encaged. If this structure were present, then it is known from clathrate studies that ten non-bonded hydrogens would extend outward from the cage.

In a mass spectrometry study of water–trimethylamine clusters, Wei et al (1991) found that the maximum number of trimethylamines that can bond to an $(H_2O)_{21}H^+$ cluster is ten, indicating that the pentagonal dodecahedron is present.

The same group carried out a similar experiment in which methanol was introduced into the system. Methanol can replace the water molecules in the $(H_2O)_{21}H^+$ cluster only where the CH_3 group can extend outward from the cage, so it was expected that a maximum of ten methanol molecules would replace water molecules in a single cluster. It was found that this was the case, as $(H_2O)_n(CH_3OH)_mH^+$ showed maximum intensity at n + m = 21 for m = 1–9 (Shi et al 1992).

Other cavity types found in clathrate hydrates have also been found as clusters in the gaseous phase. Selinger & Castleman (1991) found that magic numbers were exhibited in clusters surrounding small ions such as Cs^+, corresponding to $Cs^+(H_2O)_{20}$, but also found magic numbers at 22, 24, 27 and 29 water molecules. All these systems can be rationalised using clathrate-type cages involving 5- and 6-membered rings of water molecules. $Cs^+(H_2O)_{24}$ corresponds to the large cavity in structure-I clathrates, and $Cs^+(H_2O)_{27}$ corresponds to the large cavity in structure-II clathrates with Cs^+-H_2O enclosed.

Similar structures have been observed around other small ions such as K^+, Li^+, Na^+ and Rb^+ (Steel et al 1995).

Ionic clathrates have been observed by mass spectrometry from aqueous solution using laser-induced liquid-beam ionisation/desorption (LILBID) techniques (Sobott et al 1999) around K^+, Cs^+ and ammonium ions. The largest peak was observed at $(H_2O)_{20}$ as has been demonstrated above. Other peaks were observed corresponding to other clathrate cages encaging ions or ion–water pairs, but the ammonium ion showed a single peak at $NH_4^+(H_2O)_{20}$, corresponding to the pentagonal dodecahedron.

Biological systems

Crystallographic studies have revealed a wide range of hydration processes in biological macromolecules, ranging from neo-clathrates down to single hydrogen-bonded waters. The formation of the more complex hydrates may give some insight into the processes occurring in solution.

The most direct method of examining the hydration of a macromolecule is by using X-ray or neutron diffraction at a resolution better than 1.8 Å. In the crystal structures of proteins the macromolecules are heavily hydrated; 20–90 % of the total volume is water. The amino-acid atoms at the periphery of the molecules display larger thermal motion than those in the interior and, as the hydrating water molecules are attached to these, they sometimes have even

larger thermal motions and this can lead to ambiguities in the analysis concerning the water positions (Soper & Phillips 1986).

In biological systems, water is in a wide range of environments. Far away from a protein, for example, water should have a structure similar to the bulk, unless the protein produces an effect comparable with that of a silica surface, in which case the effect could be felt many molecular diameters away from the macromolecule.

Ordered water molecules at protein surfaces – clusters and pentagons

Most water molecules identified from X-ray electron density maps are individually bound to the protein with one or more hydrogen bonds formed with main-chain and side-chain functional groups. Two-water chains are also found in significant numbers bound to the surface of the protein. Larger clusters occur rarely but are of special interest as hydration phenomena. Clathrate hydrates form with small guest molecules that have little or no hydrogen-bonding character. In this sense the water structure around proteins must be different as the strong hydrogen-bonding groups on the side chains will determine the initial hydration shell, and whether this shell is compatible with partial clathrate cage structures will differ markedly with subtle changes in conformation. As may be expected, in the majority of protein hydration structures, the strong hydrogen-bonding groups are not compatible with clathrate geometry, and the hydration structures are characterised by single water molecules hydrogen-bonded to the functional groups. Although a minority, arrangements of hydrogen-bonded waters resembling partial clathrate structures have been found in some biological hydration structures.

In human lysozyme, the methyl group of Ala92 is surrounded by four hydrogen-bonded water molecules in a semicircle (Blake et al 1983). These waters are buried deep within the protein. If such hydration schemes were to occur at the periphery of a protein, the waters would probably not be seen in the analyses due to thermal motion.

Two fused water quadrilaterals are found between variable domains in the Bence-Jones protein Rhe (Furey et al 1983). The rings have an edge in common and are bonded to two tightly bound water molecules, and to two peptide N-H sites. Comparable quadrilateral water structures are found in structure-H clathrates, and also in cyclodextrin hydrates.

A chain of two triangles, five quadrilaterals and one pentagon of water molecules is found in a cavity between two subunits in glutathione reductase (Karplus & Schulz 1987). The water molecules are all connected to the protein main-chain or side-chain functional groups, or to other water molecules acting as anchor points. This structure does not share any structural motifs with the clathrates, and three-membered rings are not a feature of clathrates or ices. The five-membered ring in this system has two extended sides.

Three fused water pentagons are found capping an apolar valine3 side chain of the A-chain in insulin (Baker et al 1985). They are anchored by two-ring water molecules and are further stabilised by the nearby His28 side chain. The three pentagons have near-ideal pentagonal dodecahedron geometry.

Five fused pentagons forming a clathrate-like structure are the predominant feature of the hydration of the small (MW 4720) hydrophobic plant protein, crambin (Teeter 1984). Again, these rings have very close to ideal clathrate-like geometry. The network of pentagons covers a hydrophobic patch on the surface of crambin.

The crystals of crambin diffract to at least 0.88 Å, which is a far better resolution than has been obtained with any other known protein. The analysis of insulin is based on 1.5-Å data, which is also rather better resolution than most similar studies. In both these cases, the anchor points for the water structure are very tightly defined. This suggests that there may well be structures in which clathrate-like structured water may occur, but sufficient resolution to see the structure may not be achievable.

In X-ray diffraction studies on [Phe^{4}Val^6] antamanide.12H_2O (Karle 1986), all 12 water oxygens have been located, and pentagonal water rings have been found.

Substrates can sometimes mimic the hydration water structure of enzymes. An example of this is *S. griseus* protease A, which in the native state has a cluster of water molecules associated with the active site (James et al 1980). These waters are replaced by the product tetrapeptide, Ac-Pro-Ala-Pro-Tyr, and the water cluster closely resembles the shape of the product in the hydrophilic and hydrophobic areas.

Hydration of nucleic acids

The conformation of DNA is determined by water activity that can be altered by the addition of salts. If the DNA is fully hydrated, there are about twenty water molecules per nucleotide. If the hydration is reduced, the minimum number of waters per nucleotide is 3.6. The hydration of DNA can be most simply described by two hydration shells (Cohen & Eisenberg 1968), as suggested by sedimentation equilibrium studies. The first hydration shell is impermeable to ions and does not freeze into an ice-like state. Of the twenty waters per nucleotide, 11 or 12 are directly bound to the DNA. The waters have binding affinity for phosphate, phosphodiester plus sugar oxygen atoms and functional groups of bases, in order of decreasing affinity. These waters are observed in crystal structure analyses, and are hydrogen-bonded to DNA oxygen and nitrogen atoms. The second hydration shell is permeable to cations and freezes to ice I. In this respect, this shell resembles bulk water, but as Donnan-type equilibria could have an influence on the structure of this water layer around the DNA polyelectrolyte, it is believed that this layer is subtly different from bulk water far away from the DNA. In considering the hydration of A-, B-, and Z-DNA, characteristic hydration patterns are observed which can be broadly grouped into sequence-dependent and sequence-independent motifs.

Most sequence-independent motifs can be found in the minor grooves, at the sugars and at the phosphates (Jeffrey & Saenger 1991). The water molecules can bridge sites in

the same nucleotide (e.g. purine N(7) with O(6)/N(6) groups) or they can occur between different nucleotides (e.g. between a free phosphate atom and a base atom in A-DNA). Such intranucleotide bridges usually involve only one or two waters, and can bridge between strands. The separation between adjacent base pairs (~ 3.4 Å) is comparable with the separation of water–water hydrogen bonds; the waters associated with one base pair can hydrogen bond to the water associated with the adjacent base pair. In this way, extended filaments and nets of water can be built up which will cover major and minor grooves. Hydration of the phosphate backbone is sequence independent, but is structure dependent. The different puckering modes in the three DNA structures significantly affect the phosphate–phosphate distances, and hence affect the hydration structure.

At present, there are only a few cases known of sequence-specific hydration of double helical nucleic acids. In the crystal structure analyses of the two isomorphous octanucleotides, d(GGTATACC) and d(GG^{Br}UA^{Br}UACC), the same characteristic hydration pattern is observed, comprising four fused five-membered rings of water molecules (Kennard et al 1986). It seems that the sequence TATA induces the formation of these pentagons in the major groove of A-DNA. In other sequences, such as TTAA or G/C sequences, pentagons are unlikely to form because of steric reasons, and have not been observed. An isolated 5-membered ring of water molecules has been observed hydrogen bonded to G(3) O6, G(3) N3, C(4) N4 and G(13) O6 in an A-conformation octamer of d(GTGCGCAC) (Bingman et al 1992).

A spine of hydration has been observed in the minor groove of B-DNA in the central AATT sequence of the dodecamer d(CGCGAATTCGCG) (Kopla et al 1983). The waters span O(2) and N(3) atoms of bases in adjacent base pairs. These water molecules form the first hydration layer, but are connected to waters in the second hydration layer so that each water in the first layer is tetrahedrally coordinated. The N(2) amino groups in G/C interfere sterically with this regular structure; it is disrupted at both ends of the AATT sequence. In sequences other than A/T, the minor groove is wider and adjacent nucleotides are bridged by intra-chain waters between O(4′) and either purine-N(3) or pyrimidine-O(2). A further X-ray diffraction study of the same system (Tereshko et al 1999) resolved the water positions, showing four fused water hexagons dissecting the central portion of the minor groove, with the inner corners of the hexagons coinciding with the original spine water positions, and so it may be more appropriate to refer to this as a ribbon of hydration.

A spine of hydration is also observed in the minor groove of Z-DNA (Chevrier et al 1986), in which the water molecules are primarily hydrogen bonded to the O(2) atoms of the cytosine bases. These are cross-linked and there are additional water molecules bridging these waters with the phosphate oxygen atoms and with the guanine N(2) amino group.

In a room temperature X-ray diffraction study of a deoxynucleotide phosphate d(CpG)-proflavine complex.$27H_2O$ (Niedle et al 1980), most water oxygens were located, showing a hydrogen-bonding scheme involving a system of four pentagonal rings. In a low-temperature study on the same complex (Schneider et al 1992) at -2°C and -130°C, similar structures were observed, but additional water molecules were located. Nine of the waters bonded only with other waters, and these allowed the formation of water pentagons in the structure, which formed new polyhedra, giving an infinite tetrahedrally coordinated three-dimensional water network. This network was mainly constructed of pentagons, but also included 6- and 7-membered rings.

Other biological molecules

A vitamin B_{12} coenzyme ($C_{72}H_{100}CoN_{18}O_{17}P$) was analysed at 279 K in a combined X-ray and neutron diffraction experiment in which more than 140 water oxygens and 4 acetones were located (Savage et al 1987). The hydrogen-bonding scheme was disordered and an unambiguous representation of the water structure was impossible. A further study at 15 K, using a high-resolution (0.9 Å) neutron data set (Bouquiere et al 1994), showed two discrete water networks, each made up of 17 waters. One is a channel comprising statically disordered water molecules, and leading into this channel is a pocket region of highly ordered water molecules. This pocket region is mainly made up of five-membered rings in geometries concordant with clathrate structures. Although the channel region is disordered, the water molecules are arranged tetrahedrally in geometries similar to ices and clathrates.

Cyclodextrin hydrate

In the crystal structure of α-cyclodextrin hexahydrate, the hydrogen-bonding scheme has infinite chains extending throughout the crystal, and cyclic motifs are present, consisting of 4-, 5- and 6-membered hydrogen-bonded rings. Both the water molecules and the O—H groups of the cyclodextrin participate in these rings (Saenger 1979). This crystal structure has homodromic rings throughout, except where a water has to donate two hydrogen bonds to the same ring. This shows clearly that cooperative effects may be important in stabilising hydrogen-bonded rings. Rings in which the hydrogen bonds are randomly oriented (heterodromic) do not occur in any cyclodextrin hydrate structures.

Cryoprotective agents

Sugars, polyhydric alcohols and oligosaccharides are widely used as excipients in solid pharmaceutical preparations involving proteins and liposomes (Crowe et al 1996a). They are used not only because of their compatibility with, for example, proteins, but also because they are reluctant to crystallise during the drying process (Aldous et al 1995) and so increase the stability of the biomolecule.

Trehalose is found widely in plants and animals and performs a protective role, allowing survival during periods of total dehydration (Crowe et al 1996b). The exact mechanism by which trehalose exerts its protective effect is not fully known, but it is thought that the extensive hydrogen-

bonding functionality allows it to replace water molecules in the biological membranes (Miller et al 1997). This increases resistance to air-drying, and also to freeze-drying, as the number of water molecules present will be reduced and their arrangements around the trehalose molecules will prevent the formation of an extensive ice lattice. Many cryoprotective solutions have been shown to produce a glass on cooling, and a stable, wholly amorphous, state on warming (Baudot et al 2000), and this is thought to relate to their cryoprotective function. A study of trehalose solutions by Raman spectroscopy and viscosity measurements (Branca et al 1999) suggested that trehalose promotes a destructuring effect on the tetrahedral hydrogen-bond network of pure water, imposing on adjacent water molecules positions and orientations incompatible with the crystallization process.

The structural and functional features of antifreeze proteins enable them to protect living organisms by depressing freezing temperatures, modifying or suppressing ice-crystal growth, inhibiting ice recrystallization and protecting cell membranes from cold-induced damage (Fletcher et al 1999). The proteins, which are found in northern cod and antarctic fish, are up to 500 times more effective at lowering the freezing temperature than any other known solute molecule because of unique aspects of their tertiary structures, and act by specifically adsorbing to the surface of ice crystals as they form, thereby preventing their growth (Fletcher et al 2001).

Implications for drug–receptor interactions

As has been demonstrated from both experiment and computer modelling, water is composed of tetrahedral molecules which, when bonded to each other, tend to promote further bonding. The most stable resulting structures are rings and there is considerable evidence for the formation of higher-order assemblies which have pronounced geometric shapes in repeating motifs.

In considering the role of water in drug–receptor interactions, it is clear that a model that regards this solvent as a continuum is inadequate since water does more than simply separate the solute molecules. Formation of a hydrogen bond between a drug molecule and water will polarise the water, resulting in further hydrogen bonding to other water molecules. In many cases, geometry permitting, this will lead to the formation of at least one ring, which is likely to persist longer than other structures. It is apparent that the drug will tend to be hydrogen bonded with a pattern of water molecules that may play a role in receptor identification. Even in the absence of hydrogen bonding, non-polar drug molecules will tend to cause water structuring – formation of partial or total cages – which again means that the molecule will have an identifiable water 'signature'.

In the vast majority of cases, the drug will have a combination of hydrogen-bonding and non-polar groups, which will tend to give it a unique secondary identity when surrounded by water: it is possible that the primary recognition process is through the water surrounding the drug and the water surrounding the receptor. One consequence of this idea is that a drug substituent does not need to come into direct contact with the receptor to affect recognition; even when pointing outwards, towards the bulk solvent, a non-polar group which is too large may disrupt a water cage and change the recognition face. Similarly, a destructuring substituent will have a dramatic effect on the surrounding water, as will a polar group which is hydrogen-bonding but not destructuring.

The simplest kind of ligand–'receptor' interaction, which is almost entirely non-specific, is the denaturation of proteins and peptides by structure breakers such as urea and guanidinium. Denaturation is primarily a change of 3-dimensional geometry and does not require breaking of covalent bonds. The effect can sometimes be reversed by addition of a structure maker, such as sulfate. Both these effects are most easily explained by an effect on the water surrounding the macromolecule, primarily in regions where there is water structuring, which may hold the shape by virtue of 'hydrophobic bonding' between non-polar parts of the molecule. The driving force for 'hydrophobic bonding' is normally assumed to be the gain in entropy when structured water is liberated between two hydrophobic regions. Addition of urea or guanidinium disrupts the water structure and removes the driving force for close association of hydrophobic residues.

It is normally accepted that the binding of a drug to its receptor is mediated by ion–ion interactions, hydrogen bonding, dipole–dipole interactions, lipophilicity and shape complementarity (the latter being an optimisation of the first four effects), although the relative contribution of each is poorly understood. It has been shown that the optimisation of hydrophobic interactions can produce tight binding, even at the expense of possible hydrogen bonds, and this plays a large part in the 'induced fit' of receptors to ligands, allowing molecules of apparently different shapes to bind to the same part of the same receptor (Williams et al 1993). By the same token, the drug molecule may also be coerced into a certain conformation by the receptor protein (Davis & Teague 1999). These concepts are probably sufficient to account for the biological effects of many antagonists of natural ligands, since tight binding is a prerequisite to passive receptor occupancy. However, where the ligand induces a conformational change in the receptor, sufficient to trigger a biological response, passive receptor occupancy is insufficient. In this case, it may be useful to analyse the interaction as a specialised example of denaturation.

An agonist is recognised by the receptor and provokes a conformational change in the macromolecule. The change in conformation and possibly the recognition are likely to be mediated through the water which surrounds both partners. Receptor agonists such as adrenaline (epinephrine; Figure 9A) and 5-hydroxytryptamine (Figure 9B) have identifiable recognition sites around the amino group and structure-breaking moieties on the benzene ring. It is not difficult to perceive an interaction that depends on the structure-breaking effects of the catechol or phenol moiety to induce a change in receptor geometry. In comparison, analogues of the neurotransmitters that have no phenolic

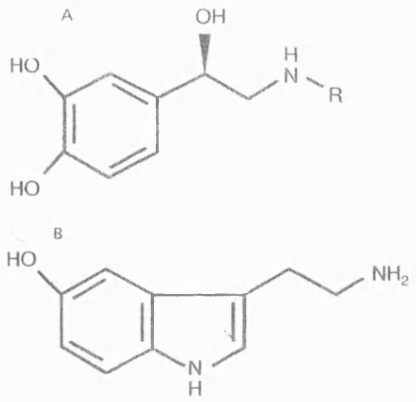

Figure 9 The structure of adrenaline (epinephrine; R = CH_3) and noradrenaline (norepinephrine; R = H) (A) and 5-hydroxytryptamine (B).

or similar structure-breaking groups will potentially be unable to induce the required conformational change and will, at best, be antagonists. It should be noted that this concept does not require the structure-breaking moiety to fit the receptor, merely to be brought into fairly close proximity, and that the effect is essentially directed outwards, towards the solvent sheath. If this idea is correct, it will be necessary, in designing a bioactive molecule, to take note of the parts of the molecule which are not in direct contact with the receptor and to explore the effect of structural changes in those regions; these changes may not only affect biodistribution and metabolism.

There are likely to be at least four ways in which water may influence the structure of a biological macromolecule (Figure 10). The first is simple bridging between two hydrogen-bonding substituents; there are several examples where this is known to occur and such effects are normal. The second is known as hydrophobic bonding, where two or more non-polar regions come together, releasing the water that is structured around both. The third involves a polar residue that is geometrically incompatible with structured water, in which case the effect may be transmitted by water molecules to neighbouring groups, disrupting hydrophobic bonding. The fourth, which does not appear to have been discussed elsewhere, would involve the connection of two domains of structured water, where these are geometrically compatible. This could occur over a relatively long range, by analogy, with the water at interfaces. Such interactions could occur in the water between non-polar groups, or could be promoted by, for example, phosphate residues: introduction of these by 'kinases' has a profound effect on the structure and function of proteins.

A vast amount of effort has been devoted to elucidation of the interactions of water with various solutes. The weight of evidence points strongly to the preference for water molecules to bond together in structures, allowing the O—O bond distance and the O—H—O bond angle to be maintained close to their optimum values. Water 'bucky

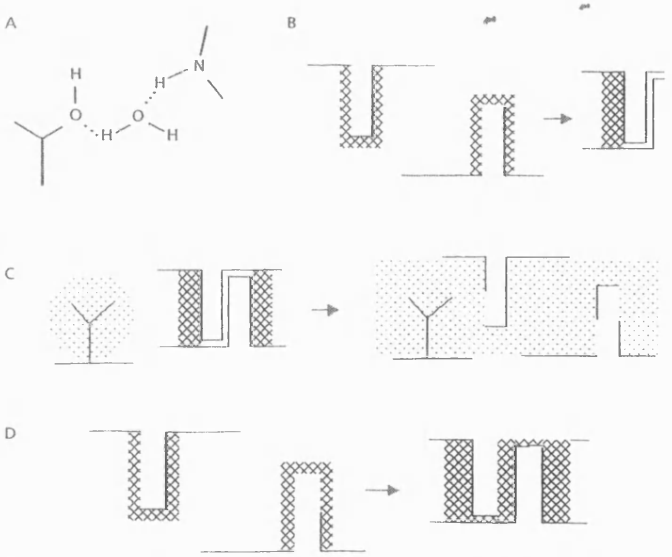

Figure 10 Types of water interaction possibly important in biological processes. A. Water bridging two hydrogen-bonding sites; note that the water is more likely to bridge between a hydrogen-bond donor and an acceptor. B. Hydrophobic effects hold the two substituents together. C. Structure-breaking substituent disrupts the hydrophobic interaction. D. Structured water between two groups holds them together.

balls', for example, are found very frequently in gas pipelines and inside mass spectrometers. However, water is not a continuum: there are regions of structuring and destructuring, dictated largely by the geometry of the system, particularly the relative disposition of functional groups on solutes. If these ideas are to be pursued, modelling of ligand–receptor interactions will have to include specific geometric considerations related to the associated water structure. This does not mean that the water around drug molecules has to be 'frozen', merely that certain structures may be favoured in a rapidly changing dynamic system, in the same way that certain conformations of the drug molecule may be favoured.

References

Aldous, B. J., Auffret, A. D., Franks, F. (1995) The crystallisation of hydrates from amorphous carbohydrates. *Cryo. Lett.* **16**: 181–186

Appenzeller, T. (1991) Fire and ice under the deep sea floor – vast undersea deposits of gas hydrates may play a major role in climate change and the future energy economy. *J. Inclus. Phenom. Mol.* **11**: 97–101

Atwood, J. L., Hamada, F., Robinson, K. D., Orr, G. W., Vincent, R. L. (1991) X-ray diffraction evidence for aromatic π hydrogen bonding to water. *Nature* **349**: 683–684

Baker, T., Dodson, T., Dodson, G., Hodgkin, D, Hubbard, R. (1985) In: Moras, D., Drenth, J., Strandberg, B., Suck, D., Wilson, K. (eds) *Crystallography in molecular biology*. New York, Plenum Press, p. 179

Barnes, P., Finney, J. L. (1979) Cooperative effects in simulated water. *Nature* **282**: 454–459

Baudot, A., Alger, L., Boutron, P. (2000) Glass forming tendency in the system water – dimethyl sulphoxide. *Cryobiology* **40**: 151–158

Ben-Naim, A. (1971) Statistical mechanical study of hydrophobic interaction. I. Interaction between two identical nonpolar solute particles. *J. Chem. Phys.* **54**: 1387–1404

Benoit, M., Bernasconi, M., Parrinelio, M. (1996) A new high-pressure phase of ice. *Phys. Rev. Lett.* **76**: 2934–2939

Bernal, J. D., Fowler, R. H. (1933) A theory of water and ionic solution with particular reference to hydrogen and hydroxyl ions. *J. Chem. Phys.* **1**: 515–548

Beuhler, R. J., Friedman, L. (1982) A study of the formation of high molecular weight water cluster ions (m/w less than 59000) in expansion of ionised gas mixtures. *J. Chem. Phys.* **77**: 2549–2557

Bhattacharyya, K., Bagchi, B. (2000) Slow dynamics of constrained water in complex geometries. *J. Phys. Chem. A* **104**: 10603–10613

Bingman, C., Li, X., Zon, G., Sundaralingam, M. (1992) Crystal and molecular structure of d(GTGCGCAC) – investigation of the effects of base sequence on the conformation of octamer duplexes. *Biochemistry* **31**: 12803–12812

Blake, C. C. F., Pulford, W. C. A., Artymiuk, P. J. (1983) X-ray studies of water in crystals of lysozyme. *J. Mol. Biol.* **167**: 693–723

Blanchard, K. C. (1940) Water: free and bound. *Cold Spring Harbour Symp. Quant. Biol.* **8**: 1–26

Blokzijl, W., Engberts, J. B. F. N. (1993) Hydrophobic effects. Opinions and facts. *Angew. Chem. Int. Ed. Engl.* **32**: 1545–1579

Boeyens, J. C. A., Oosthuizen, E.-L. (1994) The disordered macrocycle (15-ane N-5) $Cu(II)(ClO_4)_{(2)}$ at low temperature. *J. Chemical Crystallogr.* **24**: 815–816

Bouquiere, J. P., Finney, J. L., Savage, H. F. J. (1994) High-resolution neutron study of vitamin B_{12} at 15 K: solvent structure. *Acta Cryst.* **B50**: 566–578

Bowron, D. T., Filipponi, A., Roberts, M. A., Finney, J. L. (1998a) Hydrophobic hydration and the formation of a clathrate hydrate. *Phys. Rev. Lett.* **81**: 4164–4167

Bowron, D. T., Filipponi, A., Lobban, C., Finney, J. L. (1998b) Temperature-induced disordering of the hydrophobic hydration shell of Kr and Xe. *Chem. Phys. Lett.* **293**: 33–37

Bowron, D. T., Finney, J. L., Soper, A. K. (1998c) The structure of pure tertiary butanol. *Mol. Phys.* **93**: 531–543

Bradl, S., Lang, E. W. (1993) Hydration water dynamics in undercooled aqueous solutions of hydrophobic ions. *J. Phys. Chem.* **97**: 10463–10471

Branca, C., Magazu, S., Maisano, G., Migliardo, P., Villari, V., Sokolov, A. P. (1999) The fragile character and structure-breaker role of alpha, alpha-trehalose: viscosity and Raman scattering findings. *J. Phys. Condens. Matter* **11**: 3823–3832

Bruni F., Ricci, M. A., Soper, A. K. (1998) Water confined in Vycor glass. I. A neutron diffraction study. *J. Chem. Phys.* **109**: 1478–1485

Bruni, F., Ricci, M. A., Soper, A. K. (2001) Structural characterization of NaOH aqueous solution in the glass and liquid states. *J. Chem. Phys.* **114**: 8056–8063

Chaplin, M. F. (2000) A proposal for the structuring of water. *Biophys. Chem.* **83**: 211–221

Chatterjee, D. K., Seal, B. K. (1992) Studies on solute-solvent interactions in aqueous solution of isomeric mono-hydroxybenzoate salts. *Ind. J. Chem.* **A31**: 232–236

Chevrier, B., Dock, A. C., Hartmann, B., Leng, M., Moras, D., Thuong, M. T., Westhof, E. (1986) Solvation of the left handed hexamer $d(5^{Br}C\text{-}G\text{-}5^{Br}C\text{-}G\text{-}5^{Br}C\text{-}G)$ in crystals grown at two temperatures. *J. Mol. Biol.* **188**: 707–719

Claussen, W. F. (1951a) Suggested structures of water in inert gas hydrates. *J. Chem. Phys.* **19**: 259–260

Claussen, W. F. (1951b) A second water structure for inert gas hydrates. *J. Chem. Phys.* **19**: 1425–1426

Clementi, E., Brosotti, R., Fromm, J., Watts, R. O. (1976) Study of the structure of molecular complexes. XIV. Coordination numbers for selected ion pairs in water. *Theor. Chim. Acta* **43**: 101–120

Cohen, G., Eisenberg, H. (1968) Deoxyribonucleate solutions: sedimentation in a density gradient, partial specific volumes, density and refractive index measurements, and preferential interactions. *Biopolymers* **6**: 1077–1100

Collins, K. D. (1997) Charge density-dependent strength of hydration and biological structure. *Biophys. J.* **72**: 65–76

Collins, K. D., Washabaugh, M. W. (1985) The Hofmeister effect and the behaviour of water at interfaces. *Q. Rev. Biophys.* **22**: 323–422

Combariza, J., Kestner, N. R., Jortner, J. (1993) Microscopic solvation of anions in water clusters. *Chem. Phys. Lett.* **203**: 423–428

Crowe, L. M., Reid, D. S., Crowe, J. H. (1996a) Is trehalose special for preserving dry biomaterials? *Biophys. J.* **71**: 2087–2093

Crowe, J. H., Hoekstra, F. A., Nguyen, K. H. N., Crowe, L. M. (1996b) Is vitrification involved in depression of the phase transition temperature in dry phospholipids? *Biochim. Biophys. Acta* **1280**: 187–196

da Silva, F. L. B., Olivares-Rivas, W., Degreve L., Akesson, T. (2001) Application of a new reverse Monte Carlo algorithm to polyatomic molecular systems. I. Liquid water. *J. Chem. Phys.* **114**: 907–914

Dang, L. X. (1999) Characterisation of the water octamer, nanomer, decamer, and iodide-water interactions using molecular dynamics techniques. *J. Chem. Phys.* **110**: 1526–1532

Dang, L. X., Chang, T.-M. (1997) Molecular dynamics study of water clusters, liquid, and liquid-vapour interface of water with many-body potentials. *J. Chem. Phys.* **106**: 8149–8159

Darling, B. T., Dennison, D. M. (1940) The water vapor molecule. *Phys. Rev.* **57**: 128–139

Datta, A., Das, S., Mandal, D., Pal, S. K., Bhattacharyya, K. (1997) Fluorescence monitoring of polyacrylamide hydrogel using 4-aminophthalimide. *Langmuir* **13**: 6922–6926

Datta, A., Mandal, D., Pal, S. K., Das, S., Bhattacharyya, K. (1998) Solvation dynamics in organized assemblies. 4-aminophthalimide in micelles. *J. Mol. Liq.* **77**: 121–129

Davidson, D. W. (1973) Clathrate hydrates. In: Frank, F. (ed.) *Water: a comprehensive treatise*. Vol. 2, New York, Plenum Press, pp 115–234

Davis, A. M., Teague, S. J. (1999) Hydrogen bonding, hydrophobic interactions, and the failure of the rigid body hypothesis. *Angew. Chem. Int. Ed.* **38**: 736–749

Davy, H. (1811) On some of the combinations of oxymuriatic acid gas and oxygen and on the chemical relations of the properties. *Phil. Trans. Roy. Soc. (London)* **101**: 30–55

De Jong, P. H. K., Wilson, J. E., Neilson, G. W. (1997) Hydrophobic hydration of methane. *Mol. Phys.* **91**: 99–103

DeBoer, J. H., Zwikker, M. (1929) Adsorption as a result of polarisation: adsorption Isotherm. *Z. Physik. Chem.* **B-3**: 407–418

Del Bene, J. E. (1971) Theoretical study of open chain dimers and trimers containing CH_3OH and H_2O. *J. Chem. Phys.* **55**: 4633–4636

Del Bene, J., Pople, J. A. (1970) Theory of molecular interactions. I. Molecular orbital studies of water polymers using a minimal Slater type basis. *J. Chem. Phys.* **52**: 4858–4866

Derjaguin, B. V. (1966) Effect of lycophile surfaces on the properties of boundary liquid films. *Disc. Faraday Trans.* **42**: 109–119

Derjaguin, B. V. (1970) Superdense water. *Scientific American* **223**: 52–64

Ducros, P. (1960) Rotation of molecules of H_2O and the dielectric properties of the zeolites *Bull. Soc. Franc. Mineral Crist.* **83**: 85–89

Duffy, E. M., Kowalczyk, P. J., Jorgensen, W. L. (1993) Do denaturants interact with aromatic hydrocarbons in water? *J. Am. Chem. Soc.* **115**: 9271–9275

Dyke, T. R., Mack, K. M., Muenter, J. S. (1977) The structure of the water dimer from molecular beam electric resonance spectroscopy. *J. Chem. Phys.* **66**: 498–507

Echt, O., Kreisle, D., Knapp, M., Recknagel, E. (1989) Evolution of magic numbers in mass spectra of water clusters. *Chem. Phys. Lett.* **96**: 80–86

Eisenstein, A., Gingrich, N. S. (1942) The diffraction of X-rays by argon in the liquid, vapor and critical regions. *Phys. Rev.* **62**: 261–270

Eley, D. D. (1939) On the solubility of gases. Part 1: The inert gases in water. *Trans. Faraday Soc.* **35**: 1281–1293

Fang, J. X., Marlow, W. H., Lu, J. X., Lucchese, R. R. (1997) Monte Carlo studies of effects of substrate size on water-substrate interaction energy and water structure. *J. Chem. Phys.* **107**: 5212–5216

Faraday, M. (1823) On the hydrate of chlorine. *Q. J. Sci. Litt. Arts* **15**: 71–90

Fedaykin, N. N. (1962) Change of the water structure on condensation in capillaries. *Kolloid Z.* **24**: 497–503

Ferrario, M., Haughney, M., McDonald, I. R., Klein, M. L. (1990) Molecular dynamics simulation of aqueous mixtures: methanol, acetone and ammonia. *J. Chem. Phys.* **93**: 5156–5166

Filipponi, A., Bowron, D. T., Lobban, C., Finney, J. L. (1997) Structural determination of the hydrophobic hydration shell of Kr. *Phys. Rev. Lett.* **79**: 1293–1296

Finney, J. L. (2001) The water molecule and its interactions: the interaction between theory, modelling and experiment. *J. Mol. Liquids* **90**: 303–312

Fletcher, G. L., Goddard, S. V., Wu, Y. (1999) Antifreeze proteins and their genes: from basic research to business opportunity. *Chemtech.* **30**: 17–28

Fletcher, G. L., Hew, C. I., Davies, P. L. (2001) Antifreeze proteins of teleost fishes. *Ann. Rev. Physiol.* **63**: 359–390

Forslind, E., Jacobsson, A. (1975) Clay-water systems. In: Frank, F. (ed.) *Water: a comprehensive treatise*. Vol. 5, New York, Plenum Press, pp 173–248

Fowler, D. L., Loebenstein, W. V., Pall, D. B., Kraus, C. A. (1940) Some unusual hydrates of quaternary ammonium salts. *J. Am. Chem. Soc.* **62**: 1140–1144

Frank, H. S. (1958) Covalency in the hydrogen bond and the properties of water and ice. *Proc. R. Soc.* **A247**: 481–492

Frank, H. S., Evans, M. W. (1945) Free volume and entropy in condensed systems. III. Entropy in binary liquid mixtures; partial molal entropy in dilute solutions; structure and thermodynamics in aqueous electrolytes. *J. Chem. Phys.* **13**: 507–532

Frank, H. S., Wen, W. Y. (1957) Structural aspects of ion-solvent interaction in aqueous solutions: a suggested picture of water structure. *Disc. Faraday Soc.* **24**: 133–140

Franks, F. (1972–1982) *Water: a comprehensive treatise*. Volumes 1–7, London, Plenum Press

Fromm, J., Clementi, E., Watts, R. O. (1975) Coordination number for the Li^+F^- ion pair in water. *J. Chem. Phys.* **62**: 1388–1398

Furey, W., Wang, B. C., Yoo, C. S., Sax, M. (1983) Structure of a novel Bence-Jones protein (RHE) fragment at 1.6 Å resolution. *J. Mol. Biol.* **167**: 661–692

Goddard, W. A., Hunt, W. J. (1974) The Rydberg nature and assignments of excited states of the water molecule. *Chem. Phys. Lett.* **24**: 464–472

Gortner, R. A., Gortner, W. A. (1934) The cryoscopic method for the determination of 'bound water'. *J. Gen. Physiol.* **17**: 327–339

Gregory, J. K., Clary, D. C. (1996) Structure of water clusters. The contribution of many-body forces, monomer relaxation, and vibrational zero-point energy. *J. Phys. Chem.* **100**: 18014–18022

Grigorenko, B. L., Nemukhin, A. V., Topol, I. A., Burt, S. K. (2000) Hydrogen bonding at the diatomics-in-molecules level: water clusters. *J. Chem. Phys.* **113**: 2638–2674

Guillot, B., Guissani, Y. (2001) How to build a better pair potential for water? *J. Chem. Phys.* **114**: 6720–6733

Guillot, B., Guissani, Y., Bratos, S. J. (1991) A computer simulation study of hydrophobic hydration of rare gases and of methane. I. Thermodynamic and structural properties. *Chem. Phys.* **95**: 3643–3648

Haberland, H. (1984) In: Eichler, J., Vertel, I. V., Stolterfoht, N. (eds) *Electronic and atomic collisions*. Amsterdam, Elsevier, p. 597

Hall, L. (1948) The origin of ultrasonic absorption in water. *Phys. Rev.* **73**: 775–781

Halle, B., Denisov, V. P. (1998) Water and monovalent ions in the minor groove of B-DNA oligonucleotides as seen by NMR. *Biopolymers* **48**: 210–233

Hankins, D., Moskowitz, J. W., Stillinger, F. H. (1970) Water molecule interactions. *J. Chem. Phys.* **53**: 4544–4554

Hecht, D., Tadesse, L., Walters, L. (1992) Defining hydrophobicity: probing the structure of solute-induced hydration shells by fourier transform infrared spectroscopy. *J. Am. Chem. Soc.* **114**: 4336–4339

Hernandez-Cobos, J., Mackie, A. D., Vega, L. F. (2001) The hydrophobic hydration of methane as a function of temperature from histogram reweighting Monte Carlo simulations. *J. Chem. Phys.* **114**: 7527–7535

Hofmeister, W. (1888) On the understanding of the effects of salts. Second report. On the regularities in the precipitating effects of salts and their relationship to their physiological behaviour. *Naunyn Schmiedebergs Arch. Pathol. Pharmacol.* **24**: 247–260

Hori, T. (1956) Figures manifested by extremely thin film of oil. *Low Temperature Science* **A-15**: 290–297

Howell, L., Neilson, G. W. (1996) Li^+ hydration in concentrated aqueous solution. *J. Phys. Condens. Matter* **8**: 4455–4463

Ishihara, Y., Okouchi, S., Uedaira, H. (1997) Dynamics of hydration of alcohols and diols in aqueous solution. *Faraday Trans.* **93**: 3337–3342

Jalilehvand, F., Spangberg, D., Lindqvist-Reis, P., Hermansson, K., Persson, I., Sandstrom, M. (2001) Hydration of the calcium ion. An EXAFS, large-angle X-ray scattering, and molecular dynamics simulation study. *J. Am. Chem. Soc.* **123**: 431–441

James, M. N. G., Sielecki, A. R., Brayer, G. D., Delbaere, L. T. J., Bauer, C.-A. (1980) Structures of product and inhibitor complexes of streptomyces s-griseus protease at 1.8 Å resolution. A model for serine protease catalysis. *J. Mol. Biol.* **144**: 43–50

Jeffrey, G. A., Saenger, W. (1991) *Hydrogen bonding in biological systems*. Berlin, Springer-Verlag

Jeffrey, G. A., Gress, M. E., Takagi, S. (1977) Some experimental observations on H...O hydrogen bond lengths in carbohydrate crystals. *J. Am. Chem. Soc.* **99**: 609–611

Jordanides, X. J., Lang, M. J., Song, X. Y., Fleming, G. R. (1999) Solvation dynamics in protein environments studied by photon echo spectroscopy. *J. Phys. Chem. B* **103**: 7995–8005

Jorgensen, W. L., Chandrasekhar, J., Madura, J. D., Impey, R. W., Klein, M. L. (1983) Comparison of simple potential functions for simulating liquid water. *J. Chem. Phys.* **79**: 926–935

Jorgensen, W. L., Gao, J., Ravimohan, C. (1985) Monte-Carlo simulations of alkanes in water: hydration numbers and the hydrophobic effect. *J. Phys. Chem.* **89**: 3470–3474

Karle, I. L. (1986) Water structure in [Phe^{4}Val^4] antamanide.12H_2O crystallised from dioxane. *Int. J. Pept. Protein Res.* **28**: 6–12

Karplus, P. A., Schulz, G. E. (1987) Refined structure of glutathione reductase at 1.54 Å resolution. *J. Mol. Biol.* **195**: 701–729

Kauzmann, W. (1959) Some factors in the interpretation of protein denaturation. *Adv. Protein Chem.* **14**: 1–63

Kavanaugh, J. L. (1964) *Water and solute-water interactions*. San Francisco, Holden-Day

Kelkar, S. K., Selim, M. S., Sloan, E. D. (1998) Hydrate dissociation rates in pipelines. *Fluid Phase Equilibria* **151**: 371–382

Kennard, O., Cruse, W. B. T., Nachman, J., Prange, T., Shakked, Z., Rabinovich, D. (1986) Ordered water structure in a DNA octamer at 1.7 Å resolution. *J. Biomol. Struct. Dynam.* **5**: 623–648

Koehler, J. E. H., Saenger, W., LeSyng, B. (1987) Cooperative effects in extended hydrogen bonded systems involving OH groups – *ab initio* studies of the cyclic S-4 water tetramer. *J. Comput. Chem.* **8**: 1090–1098

Koga, K., Tanaka, H., Zeng, X. C. (2000) First-order transition in confined water between high-density liquid and low-density amorphous phases. *Nature* **408**: 564–567

Kopla, M. L., Fratini, A. V., Drew, H. R., Dickerson, R. E. (1983) Ordered water structure around a β-DNA dodecamer – a quantitative study. *J. Mol. Biol.* **163**: 129–146

Kristiansson, O., Lindgren, J., deVillepin, J. (1988) A quantitative infrared spectroscopic method for the study of the hydration of ions in aqueous solution. *J. Phys. Chem.* **92**: 2680–2685

Kuhne, W. (1864) Untersuchungen uber das Protoplasma. *Z. Physic. Chem. (Leipzig)* **1**: 158–164

Kusalik, P. G., Svishchev, I. M. (1994) The spatial structure in liquid water. *Science* **265**: 1219–1221

Leberman, R., Soper, A. K. (1995) Effect of high-salt concentrations on water structure. *Nature* **378**: 364–366

Levchuk, V. N., Sheyket, I. I., Simkin, B. Y. (1991) Calculation of the hydration energy of ethanol by the Monte-Carlo method. *Chem. Phys. Lett.* **185**: 339–343

Li, J. C., Ross, D. K. (1993) Evidence for two kinds of hydrogen bond in ice. *Nature* **365**: 327–329

Li, J. C., Ross, D. K., Hayes, M. I. B. (1994) The existence of 2 H-bonds of different strengths in ice and their possible implications. *J. Mol. Struct.* **322**: 131–139

Lie, G. C., Clementi, E. (1975) Study of the structure of molecular complexes. II. Structure of liquid water obtained by Monte Carlo simulation with the Hartree-Fock potential corrected by inclusion of dispersion forces. *J. Chem. Phys.* **62**: 2195–2199

Lindgren, J., Tegenfeldt, J. (1974) Hydrogen bond studies. 79. Correlation curves for properties of hydrogen bonded water molecules. *J. Mol. Struct.* **20**: 335–349

Ling, G. N. (1971) Hydration of macromolecules. In: Horne, R. A. (ed.) *Equilibrium and transport properties of water and aqueous solutions*. New York, John Wiley and Sons, pp 663–700

Liu, K., Brown, M. G., Carter, C., Saykally, R. J., Gregory, J. K., Clary, D. C. (1996) Characterization of a cage form of the water hexamer. *Nature* **381**: 501–503

Lobban, C., Finney, J. L., Kuhs, W. F. (1998) The structure of a new phase of ice. *Nature* **391**: 268–270

Luck, W. A. P. (1998) The importance of cooperativity for the properties of liquid water. *J. Mol. Struct.* **448**: 131–142

Lutz, O., Vrachopoulou, M., Groves, M. J. (1994) Use of the Walden product to evaluate the effect of amino acids on water structure. *J. Pharm. Pharmacol.* **46**: 698–703

Lyubartsev, A. P., Laasonen, K., Laaksonen, A. (2001) Hydration of Li^+ ion. An *ab initio* molecular dynamics simulation. *J. Chem. Phys.* **114**: 3120–3126

Magat, M. (1936) Raman spectrum and the constitution of liquid water. *Ann. Phys. (Paris)* **6**: 109–117

Mahoney, M. W., Jorgensen, W. L. (2000) A five-site model for liquid water and the reproduction of the density anomaly by rigid, nonpolarizable potential functions, *J. Chem. Phys.* **112**: 8910–8922

Mahoney, M. W., Jorgensen, W. L. (2001) Diffusion constant of the TIP5P model of liquid water. *J. Chem. Phys.* **114**: 363–366

Marcera, R. L., Buckingham, A. D., Skipper, N. T. (1997) The aggregation of methane in aqueous solution. *J. Chem. Soc. Faraday Trans.* **93**: 2263–2267

Marcus, Y. (1986) The hydration entropies of ions and their effects on the structure of water. *J. Chem. Soc. Faraday Trans.* **82**: 233–242

Marcus, Y. (1994) Viscosity B-coefficients, structural entropies and heat capacities and the effects of ions on the structure of water. *J. Solution Chem.* **23**: 831–848

Martinez, J. M., Pappalardo, R. R., Marcos, E. S. (1999) Shape and size of simple cations in aqueous solutions: a theoretical reexamination of the hydrated ion via computer simulations. *J. Chem. Phys.* **110**: 1669–1676

Mashimo, S. (1994) Structure of water in pure liquid and biosystem. *J. Non-Crystalline Solids* **172–174**: 1117–1120

Mashimo, S., Miura, N., Umehara, T. (1992) The structure of water determined by microwave dielectric study on water mixtures with glucose, polysaccharides and L-ascorbic acid. *J. Chem. Phys.* **97**: 6758–6765

McDonald, I. R., Klein, M. L. (1978) Molecular dynamics studies of hydrogen-bonded liquids. *Faraday Disc.* **66**: 48–57

McGreevy, R. L. (1990) Reverse Monte Carlo simulation techniques for combining neutron and X-ray diffraction data. *Inst. Phys. Conf. Ser.* **101**: 41–50

McMullan, R. K, Jeffrey, G. A. (1959) Hydrates of the tetra-n-butyl and tetra-i-amyl quaternary ammonium salts. *J. Chem. Phys.* **31**: 1231–1234

McMullan, R. K., Mak, T. C. W., Jeffrey, G. A. (1966) Polyhedral Clathrate Hydrates XI. Structure of tetramethyl ammonium hydroxide pentahydrate. *J. Chem. Phys.* **44**: 2338–2345

Miller, R. C., Anderson, R. J., Kassner, J. L., Hagen, D. E. (1983) Homogeneous nucleation rate measurements for water over a wide range of temperature and nucleation rate. *J. Chem. Phys.* **78**: 3204–3211

Miller, D., de Pablo, J., Corti, H. (1997) Thermophysical properties of trehalose and its concentrated aqueous solutions. *Pharm. Res.* **14**: 578–590

Mishima, O., Stanley, H. E. (1998) The relationship between liquid, supercooled and glassy water. *Nature* **396**: 329–335

Mootz, D., Oellers, E.-J., Wiebcke, M. (1987) First examples of type

l clathrate hydrates of strong acids. Polyhydrates of hexafluorophosphoric, tetrafluoroboric and perchloric acid. *J. Am. Chem. Soc.* **109**: 1200–1202

Morgan, J., Warren, B. E. (1938) X-ray analysis of the structure of water. *J. Chem. Phys.* **6**: 666–673

Morokuma, K., Pedersen, L. (1968) Molecular-orbital studies of hydrogen bonds. An *ab initio* calculation for dimeric H_2O. *J. Chem. Phys.* **48**: 3275–3282

Muller, N. (1990) Search for a realistic view of hydrophobic effects. *Acc. Chem. Res.* **23**: 23–28

Murphy, K. P., Friere, E. (1992) Prediction of protein stability and folding unfolding thermodynamics from crystallographic structure. *FASEB J.* **6**: A343–A343

Nageli, C. (1855) In: Nageli, C., Cramer, C. (eds) *Pflanzen-physiologische Untersuchungen* Heft. I., Schultese, Zurich. **1**: 120–138

Nakahara, M., Yoshimoto, Y. (1995) Hydrophobic slowdown and hydrophilic speedup of water rotation in supercooled aqueous solutions of benzene and phenol. *J. Phys. Chem.* **99**: 10698–10700

Nakanishi, K., Okazaki, S., Ikari, K., Touhara, H. (1981) Hydration structure around an electrolyte molecule as revealed by Monte Carlo calculation: Have you seen the iceberg? *Chem. Phys. Lett.* **84**: 428–432

Neilson, G. W., Enderby, J. E. (1982) The structure around nitrate ions in concentrated aqueous solution. *J. Phys.* **C15**: 2347–2352

Neilson, G. W., Enderby, J. E. (1983) The structure of an aqueous solution of nickel chloride. *Proc. R. Soc.* **A390**: 353–371

Neilson, G. W., Schioberg, D., Luck, W. A. P. (1985) The structure around the perchlorate ion in concentrated aqueous solutions. *Chem. Phys. Lett.* **122**: 475–479

Nemukhin, A. V., Grigorenko, B. L., Skasyrskaya, E. Y., Topol, I. A., Burt, S. K. (2000) A new hybrid approach for modeling reactions in molecular clusters: Application for the hydrogen bonded systems. *J. Chem. Phys.* **112**: 513–521

Niedle, S., Berman, H. M., Shieh, H. S. (1980) Highly structured water network in crystals of a deoxynucleoside-drug complex. *Nature (London)* **288**: 129–133

Niesar, U., Corongin, G., Clementi, E., Kneller, G. R., Bhattacharaya, D. K. (1990) Molecular dynamics simulations of liquid water using the NCC *ab initio* potential. *J. Phys. Chem.* **94**: 7949–7956

Nishikawa, K., Iijima, T. (1990) Structural study of tert-butyl alcohol and water mixtures by X-ray diffraction. *J. Phys. Chem.* **94**: 6227–6231

Nozaki, Y., Tanford, C. (1971) The solubility of amino acids and two glycine peptides in aqueous ethanol and dioxane solutions. *J. Biol. Chem.* **246**: 2211–2217

Ohmura, R., Shigetomi, T., Mori, Y. H. (1999) Formation, growth and dissociation of clathrate hydrate crystals in liquid water in contact with a hydrophobic hydrate-forming liquid. *J. Crystal Growth* **196**: 164–173

Okazaki, S., Touhara, H., Nakanishi, K. J. (1984) Computer experiments of aqueous solutions 5. Monte Carlo calculation on the hydrophobic interaction in 5 mol % methanol solution. *Chem. Phys.* **81**: 890–894

Onori, G. (1988) Adiabatic compressability and the structure of aqueous solutions of ethyl alcohol. *J. Chem. Phys.* **89**: 4325–4332

Pauling, L. (1959) The structure of water. In: Hadži, D. (ed.) *Hydrogen bonding*. London, Pergamon Press, pp 1–6

Pauling, L., Marsh, R. E. (1952) The structure of chlorine hydrate. *Proc. Natl Acad. Sci. USA* **38**: 112–118

Plumridge, T. H., Steele, G., Waigh, R. D. (2000) A geometry-based simulation of the hydration of ions and small molecules. *Phys. Chem. Comm.* **2000**: 8

Pople, J. A. (1951) Molecular theory of association in liquids. II. A theory of the structure of water. *Proc. R. Soc.* **A205**: 163–178

Powell, H. M. (1948) The structure of molecular compounds. Part IV. Clathrate compounds. *J. Chem. Soc.* **1948**: 61–73

Ravishanker, G., Mehrotra, P. K., Mezei, M., Beveridge, D. L. (1984) Aqueous hydration of benzene. *J. Am. Chem. Soc.* **106**: 4102–4108

Riter, R. E., Willard, D. M., Levinger, N. E. (1998) Water immobilization at surfactant interfaces in reverse micelles. *J. Phys. Chem.* **B102**: 2705–2714

Rousseau, D. L., Porto, S. P. S. (1970) Polywater: polymer or artifact. *Science* **167**: 1715–1719

Rubner, M. (1922) Protein metabolism with special reference to mixtures rich in carbohydrate. *Abhandl. Preuss. Akad. Wiss. Phys. Math. Kl.* **1**: 1–17

Saenger, W. (1979) Circular hydrogen bonds. *Nature* **279**: 343–344

Saenger, W. (1987) Structure and dynamics of water surrounding biomolecules. *Annu. Rev. Biophys. Chem.* **16**: 93–114

Samoilov, O. Y. (1946) Structure of aqueous electrolytes and the hydration of ions. *Zh. Fiz. Khim.* **20**: 10–19

Sato, T., Chiba, A., Nozaki, R. (1999) Dynamical aspects of mixing schemes in ethanol-water mixtures in terms of the excess partial molar activation free energy, enthalpy and entropy of the dielectric relaxation process. *J. Chem. Phys.* **110**: 2508–2521

Sato, T., Chiba, A., Nozaki, R. (2000) Hydrophobic hydration and molecular association in methanol-water mixtures studied by microwave dielectric analysis. *J. Chem. Phys.* **112**: 2924–2932

Savage, H. F. J., Lindley, P. F., Finney, J. L., Timmins, P. A. (1987) High resolution neutron and X-ray refinement of vitamin B_{12} coenzyme, $C_{72}H_{100}CoN_{18}O_{17}P.17H_2O$. *Acta Cryst.* **B43**: 280–295

Schneider, B., Ginell, S. L., Berman, H. L. (1992) Low temperature structures of DCPG-proflavine – conformational and hydration effects. *Biophys. J.* **63**: 1572–1578

Sciortino, F., Geiger, A., Stanley, H. E. (1992) Network defects and molecular mobility in liquid water. *J. Chem. Phys.* **96**: 3857–3865

Selinger, A., Castleman, A. W. (1991) Evidence for the encagement of alkali metal ions through the formation of gas-phase clathrates: Cs^+ in water clusters. *J. Phys. Chem.* **95**: 8442–8444

Senior, W. A., Verall, R. E. (1969) Spectroscopic evidence for the mixture model in HOD solutions. *J. Phys. Chem.* **73**: 4242–4249

Sharp, K. A., Madan, B. (1997) Hydrophobic effect, water structure and heat capacity changes. *J. Phys. Chem. B* **101**: 4343–4348

Shi, Z., Ford, J. V., Castleman, A. W. (1992) Clathrate structures in water-methanol clusters. *Chem. Phys. Lett.* **200**: 142–146

Shinoda, K., Kobayashi, M., Yamaguchi, N. (1987) Effect of iceberg formation of water on the enthalpy and entropy of solution of paraffin chain compounds – the effect of temperature on the critical micelle concentration of lithium perfluorooctanesulfonate. *J. Phys. Chem.* **91**: 5292–5294

Shinohara, H., Nagashima, U., Tanaka, H., Nishi, N. (1985) Magic numbers for water ammonia binary clusters – enhanced stability of ion clathrate structures. *J. Chem. Phys.* **83**: 4183–4192

Silveston, R., Kronberg, B. (1989) Water structuring around non-polar molecules as determined by HPLC. *J. Phys. Chem.* **93**: 6241–6246

Skipper, N. T. (1993) Computer simulation of methane-water solutions. Evidence for a temperature-dependent hydrophobic attraction. *Chem. Phys. Lett.* **207**: 424–429

Sobott, F., Wattenburg, A., Barth, H.-D., Brutschy, B. (1999) Ionic clathrates from aqueous solutions detected with laser induced liquid beam ionisation/desorption mass spectrometry. *Int. J. Mass Spec.* **185–187**: 271–289

Soper, A. K., Egelstaff, P. A. (1981) The structure of liquid hydrogen chloride. *Mol. Phys.* **42**: 399–410

Soper, A. K., Finney, J. L. (1993) Hydration of methanol in aqueous solution. *Phys. Rev. Lett.* **71**: 4346–4349

Soper, A. K., Phillips, M. G. (1986) A new determination of the structure of water at 25°C. *Chem. Phys.* **107**: 47–60

Starr, F. W., Harrington, S. T., Sciortino, F., Stanley, H. E. (1999)

Slow dynamics of water under pressure. *Phys. Rev. Lett.* **82**: 3629–3632

Steel, E. A., Merz, K. M., Selinger, A., Castleman, A. W. (1995) Mass spectral and computational free energy studies of alkali metal ion-containing water clusters. *J. Phys. Chem.* **99**: 7829–7836

Stillinger, F. H. (1980) Water revisited. *Science* **209**: 451–457

Sturtevant, J. M. (1977) Heat capacity and entropy changes in processes involving proteins. *Proc. Natl Acad. Sci. USA* **74**: 2236–2240

Suga, H. (1997) A facet of recent ice sciences. *Thermochim. Acta* **300**: 117–126

Tanaka, H., Nakanishi, K. (1991) Hydrophobic hydration of inert gases: thermodynamic properties, inherent structures, and normal-mode analysis. *J. Chem. Phys.* **95**: 3719–3727

Teeter, M. M. (1984) Water structure of a hydrophobic protein at atomic resolution: pentagon rings of water molecules in crystals of crambin. *Proc. Natl Acad. Sci. USA* **81**: 6014–6018

Telgmann, T., Kaatze, U. (2000) Monomer exchange and concentration fluctuations of micelles. Broad-band ultrasonic spectrometry of the system triethylene glycol monohexyl ether/water. *J. Phys. Chem. A* **104**: 1085–1094

Tereshko, V., Minasoy, G., Egli, M. (1999) A hydrat-ion spine in a β-DNA minor groove. *J. Am. Chem. Soc.* **121**: 3590–3595

Tombari, E., Ferrari, C., Salvetti, G. (1999) Heat capacity anomaly in a large sample of supercooled water. *Chem. Phys. Lett.* **300**: 749–751

Turner, J., Soper, A. K., Finney, J. L. (1990) A neutron diffraction study of tetramethylammonium chloride in aqueous solution. *Mol. Phys.* **70**: 679–700

Urahata, S., Canuto, S. (1999) Monte Carlo study of the temperature dependence of the hydrophobic hydration of benzene. *Chem. Phys. Lett.* **313**: 235–240

Vajda, S., Jiminez, R., Rosenthal, S. J., Fidler, V., Fleming, G. R., Castner, E. W. (1995) Femtosecond to nanosecond solvation dynamics in pure water and inside the gamma-cyclodextrin cavity. *J. Chem. Soc. Faraday Trans.* **91**: 867–873

van Maaren, P., van der Spoel, D. (2001) Molecular dynamics simulations of water with novel shell-model potentials. *J. Phys. Chem. B* **105**: 2618–2626

Vegiri, A., Farantos, S. C. (1993) Classical dynamics of hydrogen bonded systems – water clusters. *J. Chem. Phys.* **98**: 4059–4075

von Stackleberg, M., Muller, H. R. (1951) On the structure of the gas hydrates. *J. Chem. Phys.* **19**: 1319–1320

Wales, D. J., Hodges, M. P. (1998) Global minima of water clusters $[H_2O]_n$, $n \leq 21$, described by an empirical potential. *Chem. Phys. Lett.* **286**: 65–72

Walrafen, G. A. (1964) Raman spectral studies of water structure. *J. Chem. Phys.* **40**: 3249–3256

Walrafen, G. A. (1968) Raman spectral studies of HDO in H_2O. *J. Chem. Phys.* **48**: 244–251

Wei, S., Shi, Z., Castleman, A. W. (1991) Mixed cluster ions as a structure probe – experimental evidence for clathrate structure of $(H_2O)_{20}H^+$ and $(H_2O)_{21}H^+$. *J. Chem. Phys.* **94**: 3268–3270

Weinhold, F. (1998a) Quantum cluster equilibrium theory of liquids: general theory and computer implementation. *J. Chem. Phys.* **109**: 367–372

Weinhold, F. (1998b) Quantum cluster equilibrium theory of liquids: illustrative example to water. *J. Chem. Phys.* **109**: 373–384

Williams, D. H., Searle, M. S., Mackay, J. P., Gerhard, U., Maplestone, R. A. (1993) Towards an estimation of binding constants in aqueous solution – studies of associations of vancomycin group antibiotics. *Proc. Natl Acad. Sci. USA* **90**: 1172–1178

Woessner, D. E., Snowden, B. S. (1969) NMR doublet splitting in aqueous Montmorillonite gels. *J. Chem. Phys.* **50**: 1516–1523

Yamaguchi, T., Soper, A. K. (1999) Observation of chloride-ion hydration in high-temperature liquid and supercritical water by spherical harmonic expansion analysis. *J. Chem. Phys.* **110**: 3529–3535

Yang, X., Castleman, A. W. (1991) Laboratory studies of large protonated water clusters under the conditions of formation of noctilucent clouds in the summer mesopause. *J. Geophys. Res. – Atmospheres* **96**: 22573–22578

Yokoyama, H., Ohta, T., Iida, M. (1992) Conductometric study of ion association between tris(ethylenediamine)cobalt(III) ion and monovalent anions at temperatures from 0°C to 50°C. *Bull. Chem. Soc. Jpn* **65**: 6845–6846